# CANADIAN THESES ON MICROFICHE

# THÈSES CANADIENNES SUR MICROFICHE

National Library of Canada
Collections Development Branch

Canadian Theses on
Microfiche Service

Ottawa, Canada
K1A 0N4

Bibliothèque nationale du Canada
Direction du développement des collections

Service des thèses canadiennes
sur microfiche

## NOTICE

The quality of this microfiche is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Previously copyrighted materials (journal articles, published tests, etc.) are not filmed.

Reproduction in full or in part of this film is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30. Please read the authorization forms which accompany this thesis.

## AVIS

La qualité de cette microfiche dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

Les documents qui font déjà l'objet d'un droit d'auteur (articles de revue, examens publiés, etc.) ne sont pas microfilmés.

La reproduction, même partielle, de ce microfilm est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30. Veuillez prendre connaissance des formules d'autorisation qui accompagnent cette thèse.

THIS DISSERTATION
HAS BEEN MICROFILMED
EXACTLY AS RECEIVED

LA THÈSE A ÉTÉ
MICROFILMÉE TELLE QUE
NOUS L'AVONS REÇUE

Canada

NL 339 (r. 86/09)

National Library of Canada | Bibliothèque nationale du Canada

Ottawa, Canada
K1A 0N4

(14)

TC –

ISBN 0-315-21210-1

CANADIAN THESES ON MICROFICHE SERVICE – SERVICE DES THÈSES CANADIENNES SUR MICROFICHE

## PERMISION TO MICROFILM – AUTORISATION DE MICROFILMER

• Please print or type – Écrire en lettres moulées ou dactylographier

### AUTHOR – AUTEUR

Full Name of Author – Nom complet de l'auteur

MAH MON-MOEY

| Date of Birth – Date de naissance | Canadian Citizen – Citoyen canadien |
|---|---|
| 12.1.59 | ☐ Yes / Oui ☑ No / Non |
| **Country of Birth – Lieu de naissance** | **Permanent Address – Résidence fixe** |
| MALAYSIA | 2 Medan Tenggara<br>Penang, MALAYSIA. |

### THESIS – THÈSE

Title of Thesis – Titre de la thèse

| Degree for which thesis was presented<br>Grade pour lequel cette thèse fut présentée | Year this degree conferred<br>Année d'obtention de ce grade |
|---|---|
| MSC | 1985. |
| **University – Université** | **Name of Supervisor – Nom du directeur de thèse** |
| University of AIBERTA | Dr J.F. HENDERSON |

### AUTHORIZATION – AUTORISATION

Permission is hereby granted to the NATIONAL LIBRARY OF CANADA to **microfilm** this thesis and to **lend** or **sell copies of the film.**

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

L'autorisation est, par la présente, accordée à la BIBLIOTHÈQUE NATIONALE DU CANADA de **microfilmer** cette thèse et de **prêter** ou de **vendre des exemplaires du film.**

L'auteur se réserve les autres droits de publication; ni la thèse ni de longs extraits de celle-ci ne doivent être imprimés ou autrement reproduits sans l'autorisation écrite de l'auteur.

ATTACH FORM TO THESIS – VEUILLEZ JOINDRE CE FORMULAIRE À LA THÈSE

| Signature | Date |
|---|---|
| MMMah | 4/2/85 |



Canada

THE UNIVERSITY OF ALBERTA

# EFFECTS OF FOUR PUTATIVE INHIBITORS OF RIBONUCLEOTIDE REDUCTASE IN INTACT CELLS.

by

(C) MAH MON MOEY

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH

IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE

OF MASTER OF SCIENCE

DEPARTMENT OF BIOCHEMISTRY

EDMONTON, ALBERTA

SPRING, 1985

THE UNIVERSITY OF ALBERTA

**RELEASE FORM**

NAME OF AUTHOR MAH MON MOEY

TITLE OF THESIS EFFECTS OF FOUR PUTATIVE INHIBITORS OF RIBONUCLEOTIDE REDUCTASE IN INTACT CELLS.

DEGREE FOR WHICH THESIS WAS PRESENTED MASTER OF SCIENCE

YEAR THIS DEGREE GRANTED SPRING, 1985

Permission is hereby granted to THE UNIVERSITY OF ALBERTA LIBRARY to reproduce single copies of this thesis and to lend or sell such copies for private, scholarly or scientific research purposes only.

The author reserves other publication rights, and neither the thesis nor extensive extracts from it may be printed or otherwise reproduced without the author's written permission.

(SIGNED) MMMAL

PERMANENT ADDRESS:

2, Medan Trengganu,

Penang,

Malaysia

DATED 1st February 1985

THE UNIVERSITY OF ALBERTA

FACULTY OF GRADUATE STUDIES AND RESEARCH

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled EFFECTS OF FOUR PUTATIVE INHIBITORS OF RIBONUCLEOTIDE REDUCTASE IN INTACT CELLS. submitted by MAH MON MOEY in partial fulfilment of the requirements for the degree of MASTER OF SCIENCE.

.....

Supervisor

.....

.....

.....

Date 1st February 1985

## ABSTRACT.

The four drugs chosen for this study, *i.e.*, hydroxyurea (HU), 5-hydroxy-picolinaldehyde thiosemicarbazone (5HP), 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole (IMPY), and inosine dialdedyde (INOX) have been shown to inhibit ribonucleotide reductase in cell-free preparations; it was the aim of this project to ascertain if they inhibit ribonucleotide reductase in intact cells and if this inhibition, if it occurs, is the direct and sole cause of growth inhibition.

Inhibition of ribonucleotide reductase in intact cells would be expected to lead to lowered deoxyribonucleotide triphosphate pools, and subsequently to decreased DNA synthesis. This decreased DNA synthesis should, in turn, lead to an increase in cell volume and synchronisation of cells at late $G_1$-early S phase. In this project, the concentrations of drugs which gave approximately 20%, 40% and 60% inhibition of growth in Chinese hamster ovary cells (CHO-K1-C3), were used. The effect of the four drugs on deoxy- and ribonucleotide concentrations were determined, and the conversion of ribonucleotides into deoxyribonucleotides and thence to DNA was monitored using radioactive precursors. Effects on cell volume and cell cycle were also studied.

INOX had no effect on deoxyribonucleotide concentrations and furthermore, it synchronised cells at $G_2M$; therefore it was concluded that it does not inhibit ribonucleotide reductase in intact cells. HU and 5HP caused synchronisation of cells in S phase, whilst IMPY synchronised cells in late $G_1$-early S phases. All three drugs had an appreciable effect on deoxyribonucleotide concentrations; dATP pools were lowered and dCTP pools were increased in the presence of all three drugs, dGTP pools were increased after 8h with the highest dose of HU and 5HP and with all three concentrations of IMPY, and dTTP was relatively unchanged with HU and 5HP but increased with IMPY at 8h. In studies using radioactive precursors, DNA synthesis was found to be inhibited but radioactivity in deoxyribonucleotides was increased, generally.

Based on the discrepancies between the predicted and observed results, it was also concluded that the hypothesis that these drugs inhibit ribonucleotide reductase in intact cells is not confirmed for HU, 5HP and IMPY. However, the perturbed deoxyribonucleotide concentrations in these drug-treated cells indicate that inhibition of ribonucleotide reductase should not be completely ruled out. Of these three drugs, results of experiments using IMPY were most consistent with the predicted effects.

## ACKNOWLEDGEMENTS.

I wish to express my sincere gratitude to my supervisor, Dr. J. Frank Henderson, for his guidance, encouragement and patience thoughout the course of this work.

I also wish to thank Bonnie Gowans, Joyce Hordern-Zutter, Jean Noujaim and George Zombor for their technical assistance and for all the interesting discussions in the laboratory, to Darel Hunting and Neal Burres for advice and encouragement, and also to all the members of the McEachern Laboratories who have helped me in some way at some time during my stay.

I would also like to thank my parents who were the first to encourage me to learn and to all my friends, especially Hooi Chee, Jasmine, Minu and Pheng Siew who gave me the encouragement and inspiration to continue learning.

As a recipient of the AFHMR studentship award, I wish to thank the Alberta Heritage Foundation for Medical Research for making all this possible.

## Table of Contents


| Chapter | | Page |
|---|---|---|
| ABSTRACT. | | iv |
| ACKNOWLEDGEMENTS. | | vi |
| LIST OF TABLES | | ix |
| LIST OF FIGURES | | x |
| LIST OF ABBREVIATIONS. | | xii |
| 1. | INTRODUCTION. | 1 |
| | 1.1 Ribonucleotide Reductase. | 1 |
| | 1.2 Inhibitors of Ribonucleotide Reductase. | 8 |
| | 1.2.1 Hydroxyurea. | 9 |
| | 1.2.2 5-Hydroxy-picolinaldehyde Thiosemicarbazone. | 12 |
| | 1.2.3 2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole. | 13 |
| | 1.2.4 Inosine Dialdehyde. | 15 |
| | 1.3 Research Objectives. | 16 |
| 2. | MATERIALS AND METHODS. | 18 |
| | 2.1 Materials. | 18 |
| | 2.2 Cell Culture. | 18 |
| | 2.3 Cell Volume. | 19 |
| | 2.4 Cell Cycle. | 19 |
| | 2.5 Cell Extraction. | 20 |
| | 2.6 Ribonucleotide Concentrations. | 20 |
| | 2.7 Deoxyribonucleoside Triphosphate Concentrations. | 21 |
| | 2.8 Metabolism of Radioactive Precursors. | 22 |
| | 2.9 Chromatography. | 23 |
| | 2.9.1 Separation of Acid-soluble Nucleotides. | 23 |
| | 2.9.2 Separation of DNA Nucleosides. | 25 |

3. RESULTS and DISCUSSION. ..... 26

3.1 Growth Inhibition. ..... 26

3.2 Cell Volume. ..... 29

3.3 Cell Cycle. ..... 34

3.4 Ribonucleotide and Deoxyribonucleotide Concentrations. ..... 45

3.5 Metabolism of Radioactive Precursors. ..... 61

4. CONCLUSIONS. ..... 75

References. ..... 77

Appendix: Cell Cycle Analysis. ..... 86

## LIST OF TABLES


| Table | | Page |
|---|---|---|
| Table 1.1 | Allosteric effectors of the reduction of ribonucleotides. | 4 |
| Table 3.1 | Effect of different concentrations of HU, 5HP, IMPY and INOX on growth of CHO-K1-C3 cells after 24h treatment. | 27 |
| Table 3.2A | Ribonucleotide concentrations. | 63 |
| Table 3.2B | Deoxyribonucleotide concentrations. | 63 |
| Table 3.3 | Incorporation of radioactive adenosine into nucleotides, RNA and DNA. | 64 |
| Table 3.4 | Incorporation of radioactive guanosine into nucleotides, RNA and DNA. | 64 |
| Table 3.5 | Incorporation of radioactive cytidine into nucleotides, RNA and DNA. | 66 |
| Table 3.6 | Incorporation of radioactive uridine into nucleotides, RNA and DNA. | 66 |
| Table 3.7 | Incorporation of radioactive deoxythymidine into nucleotides, RNA and DNA. | 68 |
| Table 3.8 | Relative effects of drugs on incorporation of radioactive precursors into deoxyribonucleotides and DNA. | 69 |
| Table 3.9 | Effects of drugs on rates of radioactive dNTP incorporation into DNA. | 73 |
| Table 4.1 | Summary and Predicted and Observed Results. | 76 |

## LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| Fig. 1.1 | Deoxribonucleotide metabolism. | 2 |
| Fig. 1.2 | Chemical structures of drugs studied. | 10 |
| Fig. 3.1 | Effect of HU (A) and 5HP (B) on cell volume. | 30 |
| Fig. 3.2 | Effect of INOX (A) and IMPY (B) on cell volume. | 33 |
| Fig. 3.3 | Effect of HU on the DNA distribution histograms of CHO cells | 36 |
| Fig. 3.4 | Relative distribution of HU-treated cells in different phases of cell cycle. | 37 |
| Fig. 3.5 | Effect of 5HP on the DNA distribution histograms of CHO cells | 38 |
| Fig. 3.6 | Relative distribution of 5HP-treated cells in different phases of cell cycle. | 39 |
| Fig. 3.7 | Effect of IMPY on the DNA distribution histograms of CHO cells | 41 |
| Fig. 3.8 | Relative distribution of IMPY-treated cells in different phases of cell cycle. | 42 |
| Fig. 3.9 | Effect of INOX on the DNA distribution histograms of CHO cells | 43 |
| Fig. 3.10 | Relative distribution of INOX-treated cells in different phases of cell cycle. | 44 |
| Fig. 3.11 | Effect of HU on deoxyribonucleotide and ribonucleotide concentrations. | 47 |
| Fig. 3.12 | Deoxyribonucleotide and ribonucleotide concentrations of HU-treated cells corrected for cell volume changes. | 48 |
| Fig. 3.13 | Effect of 5HP on deoxyribonucleotide and ribonucleotide concentrations. | 50 |
| Fig. 3.14 | Deoxyribonucleotide and ribonucleotide concentrations of 5HP-treated cells corrected for cell volume changes. | 51 |
| Fig. 3.15 | Effect of IMPY on deoxyribonucleotide ribonucleotide concentrations. | 52 |
| Fig. 3.16 | Deoxyribonucleotide and ribonucleotide concentration of IMPY-treated cells corrected for cell volume changes. | 53 |
| Fig. 3.17 | Effect of INOX on deoxyribonucleotide and ribonucleotide concentrations. | 54 |
| Fig. 3.18 | Deoxyribonucleotide and ribonucleotide concentrations of INOX-treated cells corrected for cell volume changes. | 55 |

Fig. A.1 Cell cycle histograms and chi-square plots of control and drug-treated cell populations. ..... 88

## LIST OF ABBREVIATIONS.

| | |
|---|---|
| ADP | adenosine diphosphate |
| AR | adenosine |
| ATP | adenosine triphosphate |
| CDP | cytidine diphosphate |
| CHO | Chinese hamster ovary cells. |
| CR | cytidine |
| CTP | cytidine triphosphate |
| dADP | deoxyadenosine diphosphate |
| dATP | deoxyadenosine triphosphate |
| dCDP | deoxycytidine diphosphate |
| dCMP | deoxycytidine monophosphate |
| dCTP | deoxycytidine triphosphate |
| dGDP | deoxyguanosine diphosphate |
| dGTP | deoxyguanosine triphosphate |
| DNA | deoxyribonucleic acid |
| dNTP | deoxyribonucleoside triphosphate |
| dpm | disintegrations per minute |
| dTDP | deoxythymidine diphosphate |
| dTMP | deoxythymidine monophosphate |
| dTTP | deoxythymidine triphosphate |
| dUDP | deoxyuridine diphosphate |
| dUMP | deoxyuridine monophosphate |
| 5HP | 5-hydroxy picolinaldehyde thiosemicarbazone |
| HU | hydroxyurea |
| GDP | guanosine diphosphate |

| | |
|---|---|
| GR | guanosine |
| GTP | guanosine triphosphate |
| HPLC | high performance liquid chromatography |
| IMPY | 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole |
| INOX | inosine dialdehyde |
| PCA | perchloric acid |
| PEI | polyethyleneimine |
| poly[d(A-T)] | poly-deoxy (adenylate: thymidylate) |
| poly[d(I-C)] | poly-deoxy (inosinate: cytidylate) |
| PPO | 2,5-diphenyloxazole |
| POPOP | 1,4-di-2(5-phenyloxazolyl)benzene |
| RNA | ribonucleic acid |
| SD | standard deviations |
| TCA | trichloroacetic acid |
| TdR | deoxythymidine |
| UDP | uridine diphosphate |
| UR | uridine |
| UTP | uridine triphosphate |
| u.v. | ultraviolet |

# 1. INTRODUCTION.

The synthesis of DNA has been the focus of attention of many researchers, especially those in the field of cancer research. The knowledge accumulated to date has contributed vastly to the development of more effective cancer chemotherapeutic agents whose targets are the critical steps of the metabolic pathways associated with DNA synthesis.

A key step in DNA synthesis is the conversion of ribonucleotides to deoxyribonucleotides. The four deoxyribonucleoside triphosphates (dATP, dGTP, dCTP, dTTP) which are the substrates for DNA polymerase are present in very low concentrations in cells (*ca.* $10^{-12}$ moles per $10^6$ cells), and therefore constitute a potential limiting factor in the synthesis of DNA. Consequently the enzyme ribonucleotide reductase, which catalyses this conversion, is a rational target for anticancer drugs; this study concerns four drugs which inhibit this enyzme as well as cell growth.

## 1.1 Ribonucleotide Reductase.

Two classes of ribonucleotide reductase have been described in the literature [1,2]; these are distinguished on the basis of their substrate requirements. The first type uses ribonucleoside triphosphates and its typical source is *Lactobacillus leichmannii* (EC 1.17.4.2). It is monomeric and studies made using the purified enzyme have indicated the presence of one catalytic site and one regulatory site. *Escherichia coli* ribonucleotide reductase (EC 1.17.4.1) belongs to the second category and its substrates are ribonucleoside diphosphates. In contrast to the *L. leichmannii* enzyme, this is composed of two separable subunits, one of which has three binding sites, one substrate and two allosteric effectors; the other subunit contains two iron atoms.

Mammalian reductase, ribonucleoside diphosphate reductase (EC 1.17.4.1), is similar to the *E. coli* enzyme inasmuch as it uses ribonucleoside diphosphates as substrates (Fig. 1.1). It is made up of separable subunits: the M1 subunit to which substrates and effectors bind, and the M2 subunit which is believed to contain iron, though this has not yet been conclusively demonstrated [2]. Difficulties in obtaining a homogeneous preparation of the enzyme have

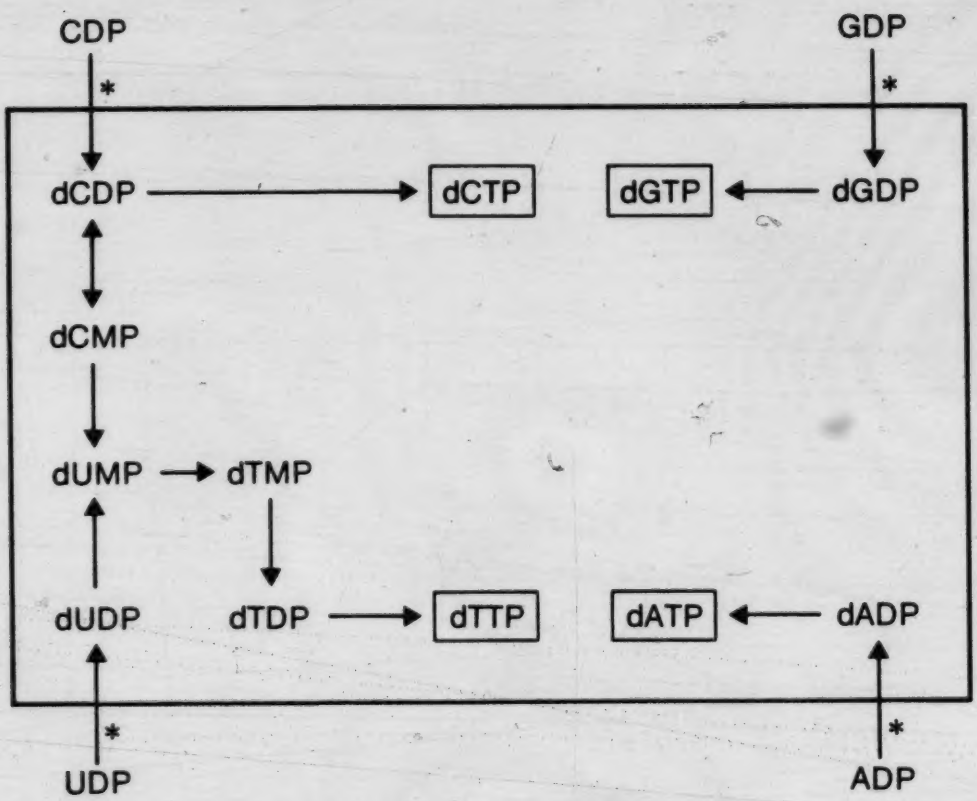



**Fig. 1.1 Deoxribonucleotide metabolism.**

hindered attempts to define precisely the structure and catalytic and regulatory mechanisms of the mammalian reductase; however Thelander and Reichard [1] have proposed that the reduction of all four substrates is catalysed by a single enzyme and that the M1 subunit of the reductase has two effector binding sites, one that regulates substrate specificity and one that regulates catalytic activity.

In order for the reduction of the ribonucleoside diphosphate substrate to take place, hydrogen donors must also participate in the reaction. Studies using purified enzymes have identified two possible reducing systems, that of thioredoxin and that of glutaredoxin; the true *in vivo* hydrogen donor is presently unknown.

Since 1966 numerous models of the catalytic mechanism and especially of the regulation of ribonucleotide reductase have been proposed. Hunting and Henderson [3] conducted a review of these models and found several common features; all models agree that dCTP has no effect on the reduction of any of the substrates, that ATP is not an inhibitor, but that dATP is always an inhibitor of ADP and GDP reduction and possibly that of CDP and UDP too. In the majority of the models, dTTP is an inhibitor of CDP and UDP reduction and a stimulator of GDP reduction. Finally, dGTP is almost always a stimulator of ADP reduction.

In the same review, Hunting and Henderson [3] included models based on studies of both mammalian and *E. coli* reductases. The mammalian reductase, like that from *E. coli*, is inhibited by dATP. Similarly, the reduction of GDP is stimulated by dTTP (or by dTTP + ATP), and the reduction of ADP is stimulated by dGTP (or by dGTP + ATP). However, in contrast to the *E. coli* reductase, reduction of CDP and UDP is stimulated by ATP. Furthermore, all mammalian reductases have an absolute requirement for the presence of a positive effector, the absence of which renders the enzyme inactive. Table 1.1 shows the effectors (inhibitors and stimulators) of the reduction of each substrate of ribonucleotide reductase.

Almost all the data obtained on the regulation of ribonucleotide reductase have come from studies using partially purified enzymes. Furthermore, these enzymological studies have

**Table 1.1. Allosteric effectors of the reduction of ribonucleotides.**

| | Substrates | | | |
|---|---|---|---|---|
| | ADP | GDP | UDP | CDP |
| Positive effectors | dGTP | dTTP | ATP | ATP |
| | dGTP + ATP | dTTP + ATP | | |
| Negative effectors | dATP | dATP | dATP | dATP |

varied only one or two effectors at a time, and have been assayed in the presence of one substrate only. One way to obtain a more accurate picture of these regulatory mechanisms would be to study the effect of varying one substrate and its effectors in the presence of all other components; however, this has not been done. Another way would be to investigate the effect of manipulating substrate and effector concentrations on ribonucleotide reductase activity in its natural environment, *i.e.*, studies using intact cells. Hunting and Henderson [4,5,8] have used this approach in their studies. Using intact Chinese hamster ovary cells (CHO), they monitored the effect of varying ribonucleotide or deoxyribonucleotide concentrations on ribonucleotide reductase activity by measuring the concentrations of the ultimate acid-soluble products of the reaction, *i.e.*, the deoxyribonucleoside triphosphates. Their data only partially agreed with the Thelander-Reichard model, one based on studies using partially purified enzyme systems; this suggests that evidence from cell-free systems does not necessarily give an accurate reflection of the state of ribonucleotide reductase in intact cells.

A third approach is the use of permeabilised cells [6,7,9]. Here the cells are made permeable to small molecules and then incubated following the addition of various effectors (ribonucleotides, deoxyribonucleotides) or drugs to the media. The advantage of such a system is that it is possible to assay ribonucleotide reductase *in situ*, assuming that permeablising the cell has no major disruptive effect on the metabolism and internal structure of the cell. However, a problem inherent in this method is that other nucleotide metabolising reactions, *e.g.*, phosphorylation or dephosphorylation of nucleoside diphosphates and triphosphates, might alter the concentrations of substrates or effectors or products of the reaction, thus giving rise to inaccurate results [6]. Hards and Wright [53] have evaluated the use of the permeabilised cell system and concluded that the presence of nucleotide metabolising reactions and changes in nucleotide pools do not present serious problems in the determination of ribonucleotide reductase activity; moreover these factors can be accounted for in the calculations. Results from work done using this system will be discussed below.

An important question raised in all studies of ribonucleotide reductase is whether the reduction of all four substrates is catalysed by a single enzyme, or by several different forms of the enzyme, or by several entirely different enzymes [3]. Traditionally, the reduction of all four substrates was believed to be carried out by a single enzyme [10,11]. This was demonstrated for ribonucleoside diphosphates using reductase from *E. coli* [10] and for ribonucleoside triphosphates using reductase from *L. leichmannii* [11]. While the issue with bacterial enzymes may appear to have been resolved in terms of one enzyme for all four substrates, questions have arisen more recently whether in the mammalian system there are specific enzymes for each of the substrates, or a different form of the same enzyme for each substrate or simply one enzyme with multiple binding sites. Cory and Mansell [12] noted that the ratio of ADP to CDP reductase activity did not remain constant in different fractions obtained during the purification procedure using Ehrlich tumor cells; moreover the response of CDP and ADP reductions to effectors (ribonucleotides, deoxyribonucleotides) and to dialdehyde derivatives of adenosine and inosine was not the same [13]. Peterson and Moore [14] also found fluctuations in the ratio of CDP to ADP reductase during the various phases of the cell cycle in synchronised Chinese hamster fibroblasts. In contrast, Cheng *et al.* [15] observed no changes in the ratio of CDP to ADP reductase throughout the cell cycle of HeLa cells or in the various fractions obtained during the purification of the reductase of Molt-4F human lymphoblasts [16]. Eriksson *et al.* [17] also found that there was little change in the ratio of CDP to GDP reductase in different fractions during the purification of calf thymus ribonucleotide reductase. Therefore, these conflicting data have not resolved the question of single enzyme versus several entirely different enzymes or multiple forms of the enzyme.

Turning to kinetic studies, Chang and Cheng [18] found that ADP and GDP, UDP and CDP acted as non-competitive inhibitors of the reduction of one another, while Cory [19] reported that UDP had no effect on CDP reduction in partially purified systems. These results do not support the one enzyme—one substrate binding site and one activator site idea of Thelander and Reichard [1], but instead imply that the mammalian reductase is more

complicated than the *E. coli* reductase.

Molecular weight determinations have also suggested differences in CDP and ADP reductases [20]. The intact enzyme and its M1 subunit have been shown to have different apparent molecular weights when assayed for ADP and CDP reductase activities. This study suggested, however, that the M2 subunit (the nonheme iron-containing subunit) was common to all four substrates. More recently, Youndale *et al.* [21,22] reported the purification of a subunit which was specific for CDP reduction and which had a molecular weight of 45,000 daltons. Additional evidence was obtained to show that iron is present in the other subunit. From their data, Youndale *et al.* proposed that the holoenzyme consists of four distinct substrate binding (M1) subunits which may be present in various combinations, plus one iron-containing (M2) subunit. Following this, Morita *et al.* [23] reported the separation of intact (M1+M2) CDP reductase from Yoshida ascites sarcoma, regenerating rat liver and mouse S-180 ascites sarcoma; this reductase showed very little activity when assayed with each of the remaining three substrates.

The most recent data regarding multiple forms of ribonucleotide reductase come from Hards and Wright [25,26]. Using permeabilised Chinese hamster ovary cells, they were able to assay for ribonucleotide reductase activity in its "physiological environment" (see above for discussion of problems associated with this method). Some of the their data agreed with the Thelander-Reichard model, *e.g.*, noncompetitive inhibition of CDP and ADP reductase activity by dATP, competitive inhibition of CDP reduction by UDP; however, other data did not, *e.g.*, noncompetitive inhibition of dGTP-activated ADP reduction by dCTP, uncompetitive inhibition of dGTP and GTP-activated ADP reduction by ATP. Also there was noncompetitive competition between dGTP and ATP for CDP reductase, and between dGTP and dATP for ADP reductase. The most significant result was that ribonucleotide reductase can simultaneously bind the nucleoside triphosphate activators for both CDP and ADP reductions. Combining their data with Youndale's [21,22] and Cory's [20] findings, Hards and Wright proposed that ribonucleotide reductase is made up of a M2 subunit which is common to the

reduction of all four substrates plus four distinct M1 substrate-binding subunits, each of which can bind two nucleoside triphosphate effectors to its specificity site at the same time, one nucleoside triphosphate to its regulatory site, and one substrate to its catalytic site. For example, the M1 subunit specific for CDP reduction would bind ATP and dCTP to its specificity site, dATP to its regulatory site, and CDP to its substrate-binding site. Although most of the data can be explained using this model, there are still some results, for example, competitive inhibition of CDP reduction by UDP, which do not fit this model. Difficulties in purifying the enzyme and problems in finding ideal assay conditions for such a highly regulated enzyme have made it difficult to resolve the question of multiple form versus single enzyme or several distinctive enzymes, although we now seem to be much closer to the answer as more homogeneous preparation of the enzyme are isolated and more data are gathered using intact cells.

Ribonucleotide reductase activity has been suggested to reflect the demand for deoxyribonucleoside triphosphates [1,2] which are used mainly in the synthesis of DNA; the level of reductase activity was much higher in regenerating rat liver [24] and rapidly growing hepatoma cells [27] compared to that of tissues with low rates of DNA synthesis. Deoxyribonucleoside triphosphates, the substrates for DNA polymerases, have been found to increase as cells progress through the S phase of the cell cycle, reaching their highest levels at late S - early $G_2M$ phase [15,28,29,30], followed by a decrease to their lowest levels in $G_1$ phase; these increases in deoxyribonucleotide concentrations were greater than 100%, and therefore are not related simply to changes in cell volume [30]. These fluctuations in levels of deoxyribonucleotides have been correlated with levels of ribonucleotide reductase activity [31].

### 1.2 Inhibitors of Ribonucleotide Reductase.

Many inhibitors of ribonucleotide reductase have been reported in the literature, of which four have been chosen for this study. These are hydroxyurea, which is in clinical use; 5-hydroxy-picolinaldehyde thiosemicarbazone, one of the most potent inhibitors reported in the

literature; 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole, a relatively new inhibitor; and inosine dialdehyde, which has been reported to affect a different subunit of ribonucleotide reductase than do the other three inhibitors.

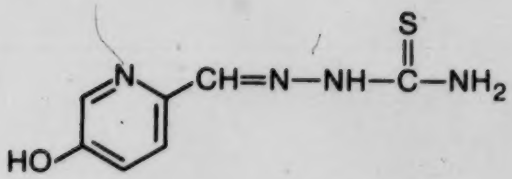
### 1.2.1 Hydroxyurea.

Hydroxyurea (HU), a simple compound structurally related to urea (Fig. 1.2), was reported in 1963 to have growth inhibitory activity against leukemia L1210 in mice; since then, this has also been demonstrated in many other experimental systems [32]. However it has not gained a prominent place in clinical cancer chemotherapy, as its effectiveness in neoplastic diseases in humans is not outstanding [33].

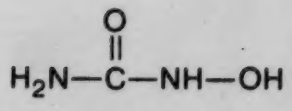
Hydroxyurea interferes with the synthesis of DNA in regenerating liver [34], in mammalian cells *in vitro* [35,36], bacteria [37,38] and echinoderm embryos [39]. In all these systems, the biological effect of hydroxyurea is partially reversible by addition of deoxyribonucleotides. As hydroxyurea inhibits DNA synthesis, it therefore affects passage of cells through the S phase of cell growth [40,41]. In contrast, RNA and protein synthesis are not affected by HU [34,38].

Mohler [36] was able partially to reverse the growth inhibition and cytocidal effects of HU in a Chinese hamster cell line (V79H) using thymidine or deoxyuridine or deoxycytidine; he therefore hypothesised that HU interfered with the conversion of ribonucleotides to deoxyribonucleotides. This hypothesis was latter confirmed by Krakoff's studies [42] using highly purified ribonucleotide reductase of *E. coli*. He showed that HU produced 70% inhibition of the *E. coli* reductase at a concentration of $3 \times 10^{-3}$ M, and that inhibition was due to inactivation of subunit $B_2$ (the equivalent of the $M_2$ iron-containing subunit in the mammalian system) by HU [43]. Studies with a mammalian enzyme (from Novikoff hepatoma cells) gave similar results [44].

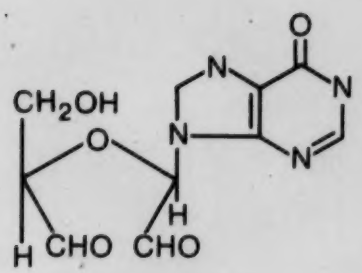
To study the action of HU in more detail, deoxyribonucleotide pool sizes were determined. It was found that addition of HU to cultured mouse embryo cells resulted in a



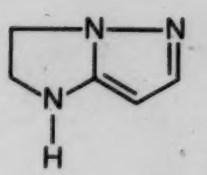
5-Hydroxy Picolinaldehyde Thiosemicarbazone (5HP)



Hydroxyurea (HU)



Inosine Dialdehyde (INOX)



2, 3-Dihydro-1*H*-imidazo [1, 2-*b*]pyrazole (IMPY)

**Fig. 1.2 Chemical structures of drugs studied.**

decrease in dATP and dGTP but an increase in dTTP and dCTP; however the turnover of dTTP was greatly diminished [45]. An increase in synthesis was thought to be the cause of this increase in dTTP and dCTP pool sizes [46]. Several researchers have also reported a decrease in dATP in hamster cells [15,29].

As mentioned briefly above, HU inhibition can be partially reversed by deoxyribonucleosides [36]. Other studies also showed partial reversal of HU inhibition, for example, inhibition of DNA synthesis in L cells by 2 mM HU was partially reversed by 0.1 mM deoxycytidine, 7 mM deoxyadenosine, 2 mM deoxyguanosine and 0.18 mM thymidine [40]; also DNA inhibition of HeLa cells produced by 0.1 - 10 mM HU was partially reversed by 0.1 mM deoxyadenosine, or by 0.1 mM deoxyguanosine and 1 mM deoxycytidine [47].

The inability to reverse HU inhibition *completely* also suggests that HU may have other possible sites of action. However, in all these reversal experiments deoxyribonucleotide concentrations were not determined, and hence the inability to reverse HU inhibition completely could be due to the failure of particular combinations and concentrations of deoxyribonucleosides to restore deoxyribonucleotides to their physiological concentrations. HU has also been suggested to have a direct effect on cellular DNA and to interact with other DNA synthesizing enzymes [38]. Skoog [45] showed that removal of HU from mouse embryo cells resulted in a rapid increase in dATP and dGTP (the two deoxyribonucleotides that were depleted in the presence of HU) but only a slow increase in DNA synthesis in spite of the fact that adequate levels of deoxyribonucleotides have been attained. More recently, HU has been implicated in inhibition of repair replication processes [48] and potentiation of chromosomal aberrations [49,50].

It can be concluded that HU does inhibit ribonucleotide reductase, as shown in studies using partially purified enzyme and by the partial recovery of activity in reversal experiments. However, it remains to be established if ribonucleotide reductase inhibition is the sole biochemical effect of HU and further studies need to be done on the other suggested effects of HU.

### 1.2.2 5-Hydroxy-picolinaldehyde Thiosemicarbazone.

The α-N-heterocyclic carboxaldehyde thiosemicarbazones constitute a class of agents which possess both antineoplastic and antiviral activity [51]. The first agent of this series to be examined for biological activity, 2-formylpyridine thiosemicarbazone, was shown to possess mild antileukemic activity against the L1210 tumor in mice [52], but only at drug levels which produced significant toxicity. Since then a variety of heterocyclic carboxaldehyde thiosemicarbazones have been synthesised and tested in a search for a more efficacious antineoplastic agent. The history of the development of the thiosemicarbazones has been reviewed by Agrawal and Sartorelli [51,53]

5-Hydroxy-picolinaldehyde thiosemicarbazone (5HP) (Fig. 1.2) is the only thiosemicarbazone that has been administered to humans in a Phase I clinical study [54]. 5HP is a formylpyridine thiosemicarbazone and was chosen for clinical study because of its high activity against transplanted tumors [53], and its relatively good solubility in water (generally, α-N-heterocyclic carboxaldehyde thiosemicarbazones are extremely insoluble). However, good activity was not observed in patients with solid tumors [54]. The relative inactivity of 5HP in humans was thought to be due to its short biological half-life.

Thiosemicarbazones inhibit the synthesis of DNA in neoplastic cells and murine neoplasms [20,21] and therefore they exert effects on the S phase of the cell cycle [57,49]. Inhibition of the biosynthesis of RNA and protein is also produced but to a lesser degree [55,56,58]. Studies of the metabolism of radioactive cytidine in intact cells showed that incorporation into the soluble deoxyribonucleotide pools was markedly depressed by 5HP while incorporation into ribonucleotides and RNA was unchanged. 5HP has also been shown to cause chromatid breaks in hamster fibroblasts, primarily during the S phase [49]. The precise mechanism(s) responsible for this fragmentation of DNA is unknown.

Interference with the biosynthesis of DNA was shown to be due to a reversible inhibition of ribonucleotide reductase [55,58,59], and in fact α-N-heterocyclic carboxaldehyde thiosemicarbazones have been reported to be the most potent inhibitors of ribonucleotide

reductase known [ref in 60]. Moore and her colleagues [58] found that 3 to 4 x $10^{-6}$ M 5HP gave 50% inhibition of the ribonucleotide reductase partially purified from Novikoff rat hepatoma.

The exact mechanism by which thiosemicarbazones inhibit enzyme activity is still not clear. Inhibition of ribonucleotide reductase by thiosemicarbazones does not seem to be influenced by its nucleoside diphosphate substrates, or by its allosteric activator, ATP. 5HP inhibition has also been shown to be partially reversed by iron and dithiothreitol (a substitute for the natural reducing substrate) [58]. A detailed investigation of the effect of iron on thiosemicarbazone inhibition of ribonucleotide reductase led to the concept of an active form of the inhibitor, *i.e.* a preformed iron chelated thiosemicarbazone [61]. This preformed iron chelate was shown to be more potent than the free inhibitor and more cytotoxic to Ehrlich ascites cells. The cytotoxicity of preformed metal chelates seems to depend on the type of metal involved. For example, the iron chelate of 5HP showed no cytotoxicity in Ehrlich ascites cells whilst the copper chelate of 5HP was cytotoxic [62]. It has been suggested that the copper chelate may have sites of action which are different from those of the iron chelate. Thelander and Graslund [63] demonstrated that it is the preformed iron chelate of thiosemicarbazones which destroys the active-site free radical of the enzyme; this leads to loss of enzyme activity.

In conclusion, inhibition of ribonucleotide reductase by 5HP appears to be due to destruction of the active-site free radical by the metal complex of the drug, and partial recovery of activity in the presence of iron and dithiothreitol can be explained in terms of restoration of this free radical. In spite of its reported potency as a ribonucleotide reductase inhibitor, however, we lack information on its effects on deoxyribonucleotide concentrations (the products of the reductase reaction).

### 1.2.3 2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole.

2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole (IMPY) was first recognised as a potential ribonucleotide reductase inhibitor when it was shown to inhibit DNA synthesis in cultured cells without affecting RNA and protein synthesis. This suggestion was later substantiated by

Brockman *et al.* [64], who demonstrated that ribonucleotide reductase activity from H. Ep.-2 cells was inhibited by IMPY. Cory and Fleischer [65] then provided an insight into the mechanism of IMPY inhibition by using separated subunits; they were able to show that IMPY inhibited the iron-containing subunit of ribonucleotide reductase from Ehrlich tumor cells. In the same paper, they also showed that IMPY inhibited the partially purified holoenzyme and that this inhibition could be partially reversed by addition of a ferrous salt, but potentiated by addition of EDTA.

It has been demonstrated by Ennis [66], that growth and DNA synthesis in L cells resume after removal of IMPY from the medium. In addition, inhibition of DNA synthesis was potentiated by the addition of deoxycytidine but protected by high concentrations of deoxyguanosine. No explanations were offered for these phenomena. More recently, Grant *et al.* [67] showed that exposure of HL-60 cells to 1 mM IMPY for 12 h produced a 40% decrease in dATP and dGTP but had no effect on dCTP and dTTP; when 3 mM IMPY was used, dATP and dGTP levels were undetectable. These findings might explain some of the effects observed by Ennis, *i.e.*, high concentrations of deoxyguanosine might help replenish depleted dGTP levels, and dGTP is a positive effector of ADP reduction (see Fig. 1.1). However, Grant's data do not explain the effect of deoxycytidine on inhibition by IMPY.

Several research groups [68,69,70,71] have reported IMPY to be an excellent cell cycle synchronizing agent. Thus HeLa cells, L1210 ascites cells and human lymphoblasts were synchronised in the late $G_1$-early S phase in the presence of IMPY. The synchronising effect of IMPY on mastocytoma cells in culture was not observed when the same cells were isolated from IMPY-treated mice [71].

As IMPY is one of the more recently studied inhibitors of ribonucleotide reductase, more work still needs to be done to rule out the possibility of other sites of action. Ennis's data from reversal experiments [66], clearly do not comply with the usual picture of inhibition of ribonucleotide reductase. and this further substantiates the need to search for a better understanding of the mechanism of inhibition of IMPY.

### 1.2.4 Inosine Dialdehyde.

Inosine dialdehyde (INOX) gained attention when it was shown to inhibit both nucleic acid synthesis in L1210 cells and tumor growth *in vivo* [72]. Its use in clinical trials led to active investigations to elucidate its mechanism of action. However, in 1981 INOX (Fig. 1.2) was withdrawn from clinical trials because of low therapeutic efficacy and toxicity [73].

Inhibition of ribonucleotide reductase by INOX was first demonstrated by Cory *et al.*, using a partially purified enzyme [72]. Ribonucleotide reductase partially purified from INOX-treated cells also exhibited a reduced level of activity. In his more recent work, Cory has shown that it was the effector binding subunit of ribonucleotide reductase that was involved in this inhibition [74,75].

INOX inhibition is thought to be irreversible [72], as the full activity of ribonucleotide reductase could not be recovered after a purification process which includes exclusion chromatography using Sephadex G-25, and ultrafiltration. However, control experiment with hydroxyurea, a known reversible inhibitor, led to full recovery of activity. In addition, it was not possible to reverse INOX inhibition of Novikoff cell proliferation by changing the medium.

In contrast to certain other known inhibitors of ribonucleotide reductase (HU, IMPY), INOX inhibited RNA and protein synthesis as well as DNA synthesis in Ehrlich tumor cells and Novikoff rat hepatoma cells [72,76]. Further studies of RNA inhibition by INOX suggested that RNA polymerase was not affected but that impairment of template function and early termination of growing RNA chains were the main causes [77]. Unlike inhibition of ribonucleotide reductase, which is thought to be due to INOX itself, RNA inhibition is thought to be caused by a triphosphate metabolite of INOX [14]. At the level of DNA synthesis, several possible sites of action have been ruled out [72]; these include thymidine kinase, dTMP kinase, dTDP kinase, thymidylate synthetase, deoxycytidylate deaminase, adenine phosphoribosyltransferase and hypoxanthine-guanine phosphoribosyltransferase.

Other effects of INOX include increased cell volume and synchronization of cells. The cell volume of Novikoff cells was shown to increase two fold when incubated with 1.66 mM

INOX for 23 hours [76], and Plageman *et al.* [76] found that INOX arrested Novikoff, L1210 and P388 cells at the $G_2$-M phase of the cell cycle. However, others have reported synchronisation at the late G1 and early S phases [78]; this difference was attributed to differences in cell type.

Summarising, INOX is the only apparently irreversible inhibitor amongst the four drugs chosen for this study; irreversibility is based on inability to recover enzyme activity after removal of drug. As RNA and protein synthesis are also inhibited, the suggested relationship between ribonucleotide reductase and growth inhibition by INOX might be different than for the other three inhibitors, and hence the degree of importance and relevance of these effects to growth inhibition need to be further investigated.

### 1.3 Research Objectives.

All four of the drugs just considered have been shown to have growth inhibitory activities and also to inhibit ribonucleotide reductase in cell-free preparations. Except in the case of HU and IMPY, it has not yet been demonstrated that they inhibit ribonucleotide reductase in intact cells as judged, for example, by reduction in deoxyribonucleoside triphosphate concentrations. It has also not been unequivocably demonstrated that these drugs are specific for ribonucleotide reductase, *i.e.*, that they have no other biochemical effects. Finally, it has not been clearly shown that the observed growth inhibition is due directly and solely to inhibition of ribonucleotide reductase. It is expected that if ribonucleotide reductase inhibition is the main biochemical effect, the concentration of one or more of the deoxyribonucleotides would decrease and this in turn should lead to inhibition of DNA synthesis and subsequently growth inhibition and cell death.

***Hence, in this project, it is my aim to test the following two hypotheses: (1) that all four drugs do inhibit ribonucleotide reductase in intact cells; and (2) that growth inhibition and inhibition of DNA synthesis is a direct and sole consequence of inhibition of ribonucleotide reductase.***

The biological system chosen for this study, is Chinese hamster ovary cells grown in suspension in deoxyribonucleoside-free α-MEM medium with 10% dialysed fetal calf serum. The deoxyribonucleotide and ribonucleotide pools of these cells and their manipulations have been studied in considerable detail [79], and the experimental study of these cells is relatively simple, technically.

Preliminary experiments were conducted to established the conditions required to produce growth inhibition, followed by studies of the effect of each of the four drugs on deoxyribonucleotide pool sizes as well as ribonucleotide pool sizes. Effects on cell volume and cell cycle changes were also monitored. Finally, radioactive precusors were used to measure the process of ribonucleotide reduction by following the conversion of the precusors to ribonucleotides and thence to deoxyribonucleotides.

## 2. MATERIALS AND METHODS.

### 2.1 Materials.

[2,8-$^3$H]Adenosine (20 Ci/mmol; 1Ci = 37 GBq), [5-$^3$H]cytidine (22 Ci/mmol), [8-$^3$H]guanosine (11 Ci/mmol), [methyl-$^3$H]thymidine (50 Ci/mmol), and [5-$^3$H]uridine (20 Ci/mmol) were obtained from Moravek Biochemicals (Brea, CA); [8-$^3$H]dATP (9.0 Ci/mmol), [5-$^3$H]dCTP (30 Ci/mmol), [8-$^3$H]dGTP (5.6 Ci/mmol) and [methyl-$^3$H]dTTP (43 Ci/mmol), were obtained from ICN Pharmaceuticals Inc. (Montreal, Que.).

DNA Polymerase I from *E. coli* was purchased from NENenzymes (Lachine, Que.), DNase I, snake venom phosphodiesterase, alkaline phosphatase, poly[d(I-C)], poly[d(A-T)], nonradioactive nucleotides and nucleosides, and mithramycin were obtained from Sigma Chemical Co. (St. Louis, MO).

PEI-cellulose plates (20 x 20cm) by Macherey-Nagel and cellulose plates by Kodak were obtained from Fischer Scientific. Co. (Edmonton, Alta.)

HU was purchased from Sigma Chemical Co. 5HP, IMPY and INOX were obtained from the Drug Synthesis & Chemistry Branch, Division of Cancer Treatment, National Cancer Institute, Bethesda, MD.

### 2.2 Cell Culture.

Chinese hamster ovary cells, CHO-K1-C3, were grown in deoxynucleoside-free α-MEM medium containing 10% dialysed fetal calf serum (Grand Island Biological Co., Grand Island, NY). CHO-K1-C3 is a subline derived from CHO-K1 cells originally obtained from Dr. G. Whitmore (Ontario Cancer Institute, Toronto, Ont.) in 1975. Cells grown in 125 mL bottles were kept in suspension using a model G-2 Gyrotary shaker (New Brunswick Scientific Co., Edison, NJ) at 200 rpm. The average doubling time was 11 ± 0.9 h and the growth rate was exponential from 1.5 x $10^4$ to 0.8 x $10^6$ - 1.0 x $10^6$ cells/mL. The cells were routinely tested for mycoplasma by the Department of Medical Bacteriology, University of Alberta, Edmonton,

Alta and found to be negative. Cell density was determined using a Model Zf Coulter Counter (Coulter Electronics Inc., Oakville, Ont.)

**2.3 Cell Volume.**

Volume distributions were determined with a Model Zf Coulter Counter fitted with a 100 μm aperture tube in conjunction with a 100-channel particle size analyzer (Channelyzer II, Coulter Electronics Inc.) interfaced with an Apple II+ computer (Apple Computer Inc., Cupertino, CA). The channelyzer was calibrated with polystyrene microspheres of 10.08 or 9.69 μm diameter (Coulter Electronics Inc., Oakville, Ont.) and volume distributions were analyzed using software obtained from Coulter Electronics Inc. A shape factor of 3/2, corresponding to a spherical non-conducting particle, was used for all determinations, and mean, median, and modal cell volumes were calculated for each distribution.

The modal cell volume was routinely utilized to represent the cell volume in both treated and untreated cell populations. The modal cell volume from untreated cultures was 1143.1 ± 169.9 fL (28 determinations). Cell volume and cell number were constant for at least 30 min following suspension in 0.15 M NaCl (data not shown). All determinations of cell volume were completed within 10-15 min following sampling.

**2.4 Cell Cycle.**

The procedure for fixing and staining cells was as follows: 2 x $10^6$ cells were centrifuged at 1000 x *g* for 5 min at 4 °C in a IEC model PR-6 centrifuge (Needham, MA) The medium was removed and cells resuspended to 2 x $10^6$ cells/mL in saline and fixed with 70% ethanol (5 mL ethanol per mL of saline) for 30 min on ice. If not used immediately, the fixed cells could be stored at 4 °C for up to two weeks. Fixed cells were centrifuged at 1000 x *g* for 2 min at 4 °C and the ethanol removed by aspiration. The cell pellet was resuspended to 2 x $10^6$ cells/mL in 100 μg/mL mithramycin (dissolved in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.1 M NaCl and 15 mM $MgCl_2$) for 30 min to quantitatively stain cellular DNA. Analysis of mithramycin

stained cells was performed using the Coulter Electronics EPICS V cell sorter (Oakville, Ont.) Excitation of mithramycin fluorescence was obtained with an argon ion laser tuned to 457 nm at a power of 200 mW. The emitted light passed through a 515 nm interference and absorbance filter, and then a 560 nm dichroic filter and a 570 nm longpass barrier filter. The data were collected by MDADS, a computerised aquisition system which is part of the EPICS V. Distribution of DNA in the different phases of the cell cycle was analysed with Coulter Electronics EASY I software (described in the Appendix) on a Terak 8510 computer interfaced with a Terak 8600 Graphics display terminal (Scottsdale, AZ).

**2.5 Cell Extraction.**

Extracts for nucleotide pool size measurements were prepared as follows: $5 \times 10^6$ - $1 \times 10^7$ cells in 50 mL centrifuge tubes were centrifuged at 1000 x g for 2 min at 4 °C in an IEC model PR-6 centrifuge (Needham, MA). The medium was removed by aspiration and the tube recentrifuged at 1000 x *g* for 5 s to remove additional medium from the centrifuge tube wall. The pellet was extracted on ice with 0.4 M PCA containing [$^3$H]adenosine for determination of dilution. After 20 min, the extract was centrifuged in a Beckman microfuge B (Palo Alto, CA) and the supernatant was removed and neutralised by extraction with 0.5 M Alamine 336 (McKerson Corp., MN) in Freon-TF (du Pont, Maitland, Ont.) [80]. Neutralised supernatants were stored at -20 °C.

**2.6 Ribonucleotide Concentrations.**

Ribonucleotide concentrations were measured by HPLC using a Waters Associates system equipped with a model 6000A chromatography pump, a U6K injector, a model 440 dual-channel detector and a Spectra-Physics autolab minigrator (Technical Marketing Assoc., Calgary, Alta.). A Partisil 10 SAX anion exchange column (Terochem, Edmonton, Alta.) was used, and nucleotides were routinely eluted isocratically at room temperature with 0.25 M $KH_2PO_4$ - 0.5 M KCl, pH 4.5, at 1.5 mL/min. This procedure allowed quantitation of ADP

and the ribonucleoside triphosphates. Detection was at 254 nm and 0.02 absorbance units full scale. The peaks were automatically integrated and the absolute amount of each nucleotide in samples was calculated on the basis of the peak area of nucleotide standards which were chromatographed frequently (twice per day). Accuracy and reproducibility of measurements using ribonucleoside triphosphate standards were 98.6% and 2.4% (SD; 3 determinations), respectively.

**2.7 Deoxyribonucleoside Triphosphate Concentrations.**

These nucleotides were determined by a modification of the DNA polymerase method [81]. The following components were common to both the dATP and dTTP assay in a final volume of 180 μL: 0.02 $A_{280}$ units poly[d(A,T)], 1.8 μmol $MgCl_2$, 1.8 μmol dAMP, 18 μmol Hepes buffer, pH 7.4, and 0.75 Richardson units of DNA polymerase I [81]. As well, the dATP assay contained 100 pmol (0.5 μCi) [$^3$H]dTTP, and 0 - 75 pmol dATP standard, while the dTTP assay contained 100 pmol (0.5 μCi) [$^3$H]dATP and 0 - 75 pmol dTTP standard.

The following components were common to both the dGTP and the dCTP assays in a final volume of 180 μL: 0.02 $A_{280}$ units poly[d(I,C)], 1.8 μmol $MgCl_2$, 1.8 μmol dAMP, and 18 μmol Hepes buffer, pH 7.4. As well, the dGTP assay contained 100 pmol (2.2 μCi) [$^3$H]dCTP, 0 - 50 pmol dGTP standard, and 1.9 units DNA polymerase I. The dCTP assay contained 240 pmol (0.5 μCi) [$^3$H]dGTP, 0 - 75 pmol dCTP standard and 3.0 units DNA polymerase I.

The reaction was started by addition of the DNA polymerase I, followed by incubation at 37 °C. At specific time points (30 min for the dGTP and dCTP assays, and 50 min for the dATP and dTTP assays), aliquots were removed and spotted on squares of Whatman 3MM filter paper. The squares were washed (3 x 15 min) with a solution of 5% TCA and 1% sodium pyrophosphate (20 mL/square), then rinsed once with 95% ethanol, and finally washed (1 x 15 min) with 95% ethanol. The filters were counted in toluene scintillation solution containing 4 g PPO and 0.1 g POPOP per litre xylene.

Results were corrected for the washing background and for effects of the dilution of the specific activity of the labeled deoxyribonucleotide by the sample with respect to both sample incorporation and the background incorporation. The calculations involved for this correction can be found in ref [79]. The accuracy and reproducibility (SD, percent) of the assays of deoxyribonucleoside triphosphate standards, as calculated from four determinations, were as follows: dATP, 95% and 13.5%, respectively; dTTP, 83% and 9.9%; dCTP, 93% and 3.3%; and dGTP, 86% and 1.3%.

**2.8 Metabolism of Radioactive Precursors.**

CHO-K1-C3 cells at a density of 200,000 to 400,000 cells/mL were incubated at 37 °C with and without drugs for 4 h. They were then incubated for 30 min with radioactive precursors at concentrations between 0.2 and 0.9 μM and at specific activities between 5.5 and 50 Ci/mmol. The cells were pelleted by centrifugation and extracted with 100 μL of ice-cold 0.4 M PCA containing [$^{14}$C]adenine for determination of dilution. After 20 min on ice, the samples were centrifuged. Supernatants were removed and neutralized using an equal volume of 0.5 M Alamine 336 in Freon-TF [80]. Ten microlitres was counted in Triton X-100 counting fluid (0.4% PPO, 0.02% POPOP, in 1 part Triton X-100 and 2 parts xylene) to measure [$^{14}$C]adenine radioactivity in order to calculate dilution occurring during extraction. Radioactivity in the extract mainly represented label incorporated into nucleotides but there was also some label from the original precursor. Neutralized extracts were stored at -20 °C. Radioactivity in individual nucleotides was determined as described below.

The acid-insoluble pellet, containing labelled DNA and RNA, was washed three times with ice-cold 0.4 M PCA, and the final wash counted in Triton X-100 to ensure complete removal of the acid-soluble fraction, The pellet was then dissolved in 200 μL 0.3 N KOH and incubated for 18 h at 37 °C to hydrolyze the RNA. After RNA hydrolysis, 10 μL DNA (1mg/mL) was added and all of the DNA content in the the hydrolysate was precipitated on ice by addition of 20 μL 4 M PCA. After centrifugation, the supernatant containing the 2'- and

3'-monophosphates from the hydrolyzed RNA was removed and neutralised with an equal volume of Alamine 336 - Freon-TF. Ten microlitres was counted in Triton X-100 counting fluid before chromatography to determine the total radioactivity in the RNA nucleotides; the remainder was stored at -20 °C.

The DNA pellet was washed three times with ice-cold 0.4 M PCA, (the final wash was counted to check that all of the RNA hydrolysate had been removed), then dissolved in 50 μL of 100 mM Tris buffer, pH 8.0. $MgCl_2$, NaCl and DNase I were added to final concentrations of 2.5 mM, 5.0 mM and 50 μg/mL, respectively, and the solution was incubated ca. 18 h at 37 °C. Ammonium acetate, $MgCl_2$, snake venom phosphodiesterase, and alkaline phosphatase were then added to final concentrations of 100 mM, 2 mM, 50 μg/mL ($10 \times 10^{-3}$ U) and 165 μg/mL (182 U), respectively, and the solution was incubated at 37 °C for ca. 18 h. If alkaline phosphatase was not used, conditions which resulted in complete DNA hydrolysis produced both the deoxyribonucleoside monophosphates and deoxyribonucleosides, complicating the separation procedure. Ten microlitres of the DNA hydrolysate was counted in Triton X-100 counting fluid to determine total radioactivity before chromatography. The DNA hydrolysate was stored at -20 °C.

## 2.9 Chromatography.

### 2.9.1 Separation of Acid-soluble Nucleotides.

A modification [79] of the method by Crabtree and Henderson [82], for separating purine and pyrimidine ribo- and deoxyribonucleotides was tested initially. Briefly, PEI-cellulose thin-layer plates, prewashed with 1.8% ammonium formate, 2% boric acid, pH 7.0, and then with 50% methanol in water were used. After application of markers, chromatograms were washed with 50% methanol in water and developed in the first dimension with 1.8 M ammonium formate, 2% boric acid, pH 7.0, followed by 3.3 M ammonium formate, 4.2% boric acid, pH 7.0. The plates were then developed in the second dimension with 0.5 M

sodium formate, pH 3.4, followed by 2.0 M sodium formate, pH 3.4, and 4.0 M sodium formate, pH 3.4, to separate the mono-, di- and triphosphates. The spots were visualised with u.v. light.

We were unable to obtain the separations obtained by Hunting [79], and various modifications of this method were therefore investigated: prewashing plates in 20% ammonium formate, 4.2% boric acid, pH 7.0, instead of 1.8% ammonium formate, 2% boric acid ; prewashing plates in 95% methanol instead of 50% methanol in water; developing in the first dimension with 0.9 M ammonium formate, 1% boric acid, pH 7.0, followed by 1.8 M ammonium formate, 2% boric acid, pH 7.0. Although some modifications gave relatively good separations on the first trial, we were not able to obtain the same separation in a second experiment. Overall, there was poor separation of ribo- from the deoxyribonucleotides and the triphosphates were streaked badly, whereas separation in the second dimension was satisfactory. It was thought that these problems could be due to the age of the plates (manufactured in 1980 and 1981). A new batch of plates (manufactured in Dec., 1982) was purchased, tested and found to be the same. This approach was then abandoned, and new methods were tested and used to separate nucleotides.

Purine nucleotides were separated by a modification of the method of Schwartz and Drach [83]. PEI-cellulose plates were attached with wicks and run overnight with 1 M LiCl in 1 M acetic acid. The wicks were then removed and the plates immersed in distilled water for 5 - 10 min and dried. Markers and samples were spotted onto the plates and fresh wicks attached. The plates were developed in the first dimension with 2 M LiCl in 1 M acetic acid until the solvent ran into the wicks (if 1981 plates were used, 1 M LiCl in 1 M acetic acid was sufficient). We found that the low pH (pH 2.0) of these solvents did not cause any breakdown of triphosphates but as a precaution, the pH of this solvent was adjusted to 3.4 with ammonia in our experiments. The wicks were removed and the plates immersed in methanol for 5 - 10 min. After drying, the plates were developed in the second dimension with 95% ethanol-2 M LiCl (1:1) saturated with boric acid and neutralised with ammonia. After the triphosphates had

been separated, the plates were dried, the spots were cut out and counted in 5 mL counting fluid. The counts obtained were corrected for recovery and counting efficiency. To do this, a standard radioactive deoxyribonucleotide was run with each batch of chromatograms.

Pyrimidine nucleotides were separated using the method by Seipel and Reichert [84]. Markers and samples were spotted onto cellulose thin layer plates. The plates were then developed in the first dimension with methanol—1-propanol—25% (w/w) ammonia — 1% (w/v) EDTA (3:9:2:6) to the middle of the plate and then dried. To complete the first dimension the plates were redeveloped in the same solvent to the top of the sheet and dried. In the second dimension, the plates were developed with isobutyric acid—25% (w/w) ammonia—1% (w/v) EDTA (200:9:114) in a single run to the top of the sheet. The spots were visualised with u.v. light and then cut out and counted in counting fluid.

### 2.9.2 Separation of DNA Nucleosides.

Markers plus samples were streaked onto cellulose thin-layer plates. Samples containing purine deoxyribonucleosides were developed in isobutyric acid—ammonium hydroxide—0.5% EDTA (200:9:14). Samples containing labelled pyrimidine deoxyribonucleosides were developed in ethyl acetate—isopropanol— water (50:18:10). After development, the plates were dried, the spots were visualised, scraped into scintillation vials, eluted with 0.5 mL 1 N NaOH for 18 h and then neutralised with 0.5 mL 1 N acetic acid. The samples were counted in 10 mL Triton X-100 counting fluid. The origins were also routinely counted to check for incomplete DNA hydrolysis.

## 3. RESULTS and DISCUSSION.

### 3.1 Growth Inhibition.

The effects of HU, 5HP, IMPY and INOX on the growth of CHO-K1-C3 cells was not known at the begining of this study, and it was therefore necessary to determine if these drugs were growth inhibitory and if so to establish the degree of growth inhibition by a range of concentrations of each of the drugs. From these results, the concentrations that gave *ca.* 20%, 40% and 60% inhibition of growth would be selected and used for further experiments. These concentrations were chosen in the expectation that any biochemical changes observed under these conditions would be in the chain of causative events leading to growth inhibition rather than being results of growth inhibition.

CHO cells were grown in 50 mL bottles for at least 18h after subculturing; this was to ensure that the cells were in log phase and had undergone one doubling (doubling time = 12h) and therefore had overcome any lag phase that might have arisen, due to subculturing. Different concentrations of drugs dissolved in saline (HU, IMPY) or media (INOX) or 70% ethanol (5HP) were added to the cultures and cell numbers were monitored at various time points; medium was used because INOX dissolved much faster in this than in saline. Due to solubility problems, 70% ethanol had to be used for 5HP. Controls contained saline or ethanol. Experiments using 70% ethanol showed that it had no effect on growth at the specific volumes used; the final ethanol concentration used was 0.08%.

**HU.** The concentration range initially tested for HU was from 10 $\mu$M to 1000 $\mu$M, followed by more growth inhibition experiments in the 50 $\mu$M to 200 $\mu$M range. The final concentrations chosen for HU were 56 $\mu$M, 100 $\mu$M and 200 $\mu$M, which produced *ca.* 25%, 40% and 55% inhibition of growth, respectively, at 24h (Table 3.1). Inhibition of growth for all three concentrations of HU was evident by 8h.

**5HP.** For 5HP, the effects of 1 $\mu$M to 100 $\mu$M doses were tested first. From these initial experiments, 2.8 $\mu$M, 5.5 $\mu$M and 10 $\mu$M concentrations were chosen for this project;

**Table 3.1. Effect of different concentrations of HU, 5HP, IMPY and INOX on growth of CHO-K1-C3 cells after 24h treatment.**

| Drug | Concentration | % Inhibition of Growth[1] |
|---|---|---|
| HU | 56 μM | 25 |
| | 100 μM | 40 |
| | 200 μM | 55 |
| 5HP | 2.8 μM | 15 |
| | 5.5 μM | 30 |
| | 10.0 μM | 60 |
| IMPY | 0.5 mM | 33 |
| | 1.0 mM | 50 |
| | 2.0 mM | 60 |
| INOX | 50 μM | 5 |
| | 100 μM | 25 |
| | 200 μM | 55 |

[1]Inhibition of growth was calculated from doubling time.

they produced *ca.* 15%, 30% and 60% inhibition of growth (Table 3.1).

**IMPY.** From the results of growth inhibition experiments using between 0.25 mM and 2.0 mM IMPY, concentrations finally decided upon were 0.5 mM, 1.0 mM and 2.0 mM (Table 3.1) which correspond to 30%, 50% and 60% inhibition, respectively.

**INOX.** With INOX, after several preliminary experiments with concentrations ranging from 10 $\mu$M to 800 $\mu$M, the following three concentrations were chosen for this project: 50 $\mu$M, 100 $\mu$M and 200 $\mu$M (Table 3.1). These concentrations produced 5%, 25% and 55% inhibition, respectively.

From the dose-response results obtained, it can be concluded that all four drugs do inhibit growth and that 5HP is the most potent agent: only 10 $\mu$M 5HP was needed to give effects similar to 200 $\mu$M HU and INOX or 2 mM IMPY. This is consistent with literature reports that thiosemicarbazones are some of the most potent ribonucleotide reductase inhibitors known. Of the four drugs studied, IMPY is the least potent drug; 2 mM was needed to obtain 60% inhibition of growth.

The results obtained were fairly reproducible throughout the two years of experimentation. For example, an experiment with HU repeated after seven months gave similar growth inhibition. An average of approximately 10% variation in percent inhibition of growth was found with these drugs and this variation was independent of time: experiments repeated after a month gave similar variations. These variations could be attributed to small changes in cell response to drugs or due to variations in technical manipulations of cells.

Sato *et al.* [85] observed that 50 $\mu$M, 100 $\mu$M and 200 $\mu$M HU gave 20%, 50% and 95% of inhibition of growth respectively, when incubated with L1210 cells for 5 days; this is similar to some of our results with HU. Sato also studied inhibition of L1210 cell growth by IMPY: he observed 15% and 45% inhibition after incubation with 0.1 mM and 0.25 mM IMPY, respectively, for five days. In experiments using HeLa cells, 60% inhibition was obtained after 24h using 0.1 mM IMPY [68], whereas higher concentrations were needed to achieve the same degree of inhibition at 24h for CHO cells. Compared to CHO cells, rat hepatoma cells were less

sensitive to INOX; 330 $\mu$M was needed to give 50% inhibition at 24h. Most of the earlier work done with 5HP, employed animal survival time experiments. However, in general agreement with these results, we found that 5HP was the most potent of the four drugs studied.

The succeeding experiments in this study were done using the concentrations of drugs mentioned earlier; all experiments were performed twice and duplicate measurements made in all experiments except for the radioactive precursor metabolism studies where triplicate measurements were made. The results reported here are representative results from two or three experiments.

### 3.2 Cell Volume.

Cell volume was observed to increase in the presence of the putative inhibitors of ribonucleotide reductase used in this study. These changes in cell volume could be the result of several kinds of biochemical changes in the cells. For example, as cells progress through the S phase they synthesize the DNA, RNA and protein necessary for cell division, and consequently cell size increases; at M phase, cells are approximately twice the size of $G_1$ cells; therefore, if cells were blocked at phases later than $G_1$, cell volume would also increase. Cell volume increases are also associated with the phenomenon of unbalanced growth. This is not fully understood, but essentially is a decrease in the rate of DNA synthesis with normal or near normal rates of RNA and protein synthesis; associated with this effect is blockade of cells at late $G_1$-early S phase. Hence it would not be surprising if cell volume increased in the presence of drugs that inhibit DNA synthesis but not RNA synthesis. A further potential cause of increased cell volume is the disrupted pumping of $Na^+$ out of cells brought about by decreased ATP concentrations.

**HU.** The modal cell volume of control and drug-treated cultures was measured as described in Chapter 2. The modal volume of HU-treated cells was increased slightly by 8h, but these increases were more evident at 16h and 24h (Fig. 3.1A) All three concentrations gave increased cell volume with increasing incubation time. The highest dose, 200 $\mu$M, resulted in a

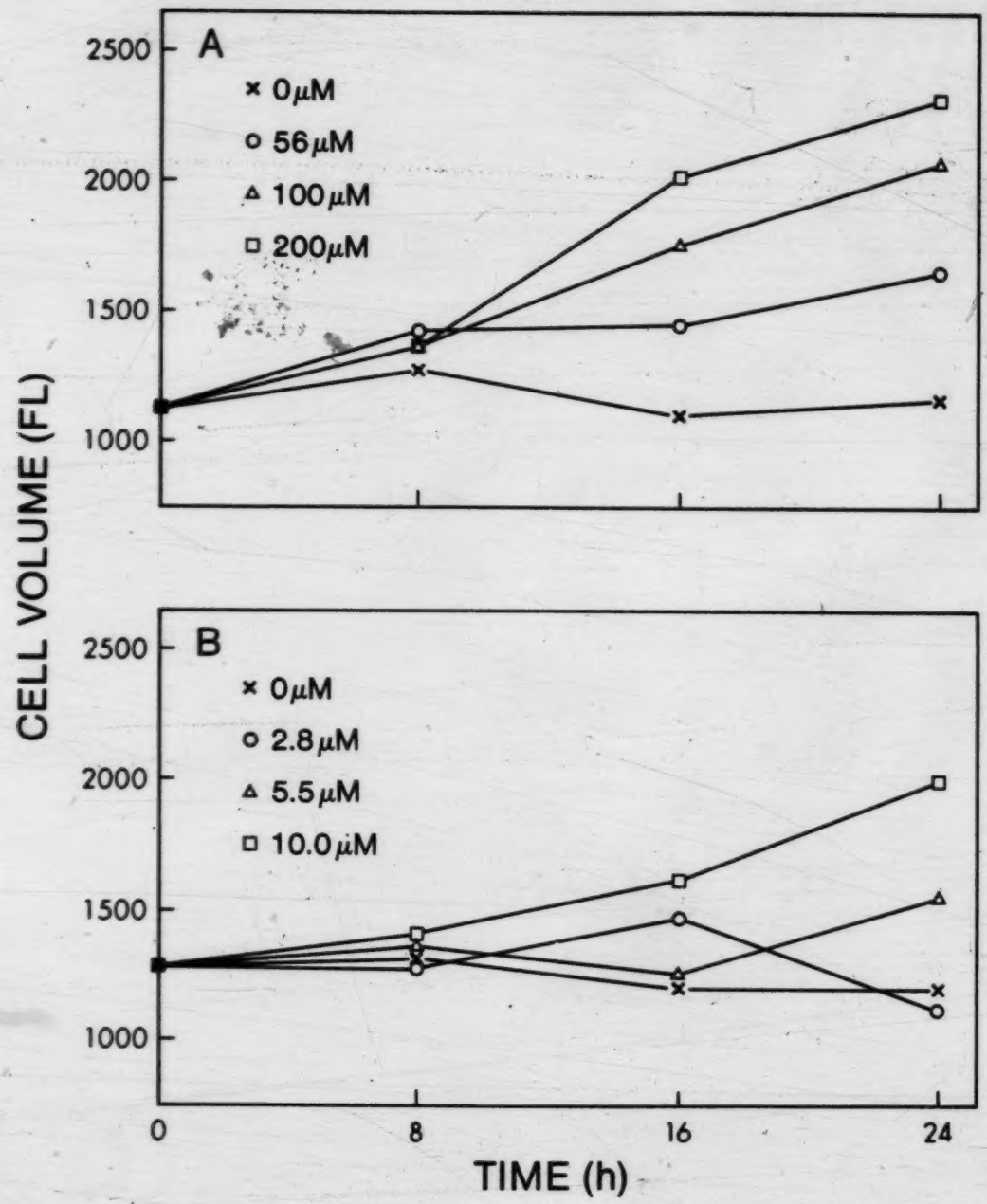



Fig. 3.1 Effect of HU (A) and 5HP (B) on cell volume.

100% increase in cell volume at 24h.

Based on results obtained in later experiments (discussed below), one can draw some tentative conclusions regarding the cause(s) of this increase in modal cell volume. First, the $Na^+$ pump suggestion could be discarded because no decrease in ATP levels was observed (results shown in next section). The suggestion that cells might be blocked at phases later than $G_1$ is not consistent with the results obtained from cell cycle analysis (where the block is at late $G_1$-early S phases). As this block at late $G_1$-early S is not complete, partially synchronised cells can still travel through the S phase, and cell volume increases as cells progress from $G_1$ through to $G_2M$ phase [89]. However, as not all of the HU-treated cells are in late S or $G_2M$, this reasoning does not completely explain the 100% increase in modal cell volume. Results from metabolism studies indicate that the unbalanced growth hypothesis is most consistent with the observed increase in modal cell volume: incorporation of radioisotopes into DNA was decreased but there was almost negligible effect on incorporation into RNA. Therefore, the concentration-dependent increase in cell volume may be explained by a concentration-dependent inhibition of DNA synthesis.

Although an increase in modal cell volume is consistent with our hypothesis that the drug inhibits ribonucleotide reductase (inhibition of ribonucleotide reductase leads to decreased DNA synthesis and consequently unbalanced growth), this is not proven because inhibition of DNA synthesis could be due to reasons other than inhibition of ribonucleotide reductase. A lack of effect or decrease in modal cell volume would not be consistent with the hypothesis.

**5HP.** Unlike HU, CHO cells exposed to 5HP did not consistently show increases in volume with time. Small increases in cell volume were observed at 8h (Fig. 3.1B); however, the volume of CHO cells exposed to 5.5 $\mu$M 5HP decreased slightly at 16h but increased to 124% of control at 24h. Cells exposed to 2.8 $\mu$M 5HP decreased in volume to control values at 24h. The highest dose of 10.0 $\mu$M produced 60% increase in cell volume at 24h, however.

The small increase in cell volume of cells exposed to 5HP could be a result of partial synchrony of cells, *i.e.*, movement of a partially synchronised population of cells through S

phase (Fig. 3.5). Again, like HU, the $Na^+$ hypothesis may be discarded because ATP concentrations were not decreased. DNA synthesis was also decreased in 5HP treated cells but, in general, to a lesser extent than HU-treated cells; cell volume increases here were also smaller. Based on the cell volume data alone, it is not possible to state whether the working hypothesis is correct for 5HP; however the data are consistent with inhibition of DNA synthesis.

**IMPY.** IMPY had effects on modal cell volume similar to those of HU. At all three concentrations used, cell volume increased with increasing incubation time (Fig. 3.2A), and incubation with 2.0 mM IMPY for 24h caused a 160% increase in cell volume.

Of the four drugs studied, IMPY gave the greatest increase in cell volume, *ca.* 160%. As with HU and 5HP, no decrease in ATP concentration was observed, and therefore the $Na^+$ pump hypothesis may be discarded. Cell cycle data show that in the presence of IMPY, cells were synchronised at late $G_1$-early S phases. However, the passage of a partially synchronised population of cells through S would not explain the 160% increase in modal cell volume. The most likely reason for this increase is unbalanced growth; of the four drugs studied, in general IMPY gave the greatest inhibition of DNA synthesis; therefore, we assume that a greater degree of unbalanced growth is taking place which is reflected in the size of the cell. Based on this argument, we suggest that our result is consistent with the hypothesis that IMPY inhibits ribonucleotide reductase in intact cells.

**INOX.** In contrast to HU and IMPY, INOX had the least effect on cell volume. The highest dose of 200 $\mu$M only produced a 40% increase in cell volume (Fig. 3.2B).

As with the other three drugs, ATP concentration in cells were not decreased, therefore the $Na^+$ pump hypothesis does not apply. Although INOX-treated cells were blocked at $G_2M$, modal cell volume did not increase by 100%; this is because at the time of treatment the cell population was asynchronous. The increase in modal cell volume in this case was less than the increases in HU, 5HP and IMPY-treated cells; this may be explained by a smaller degree of unbalanced growth due to less inhibition of DNA synthesis. As in the case of 5HP, we are not able to state definitively if the hypothesis is correct based on the present evidence.

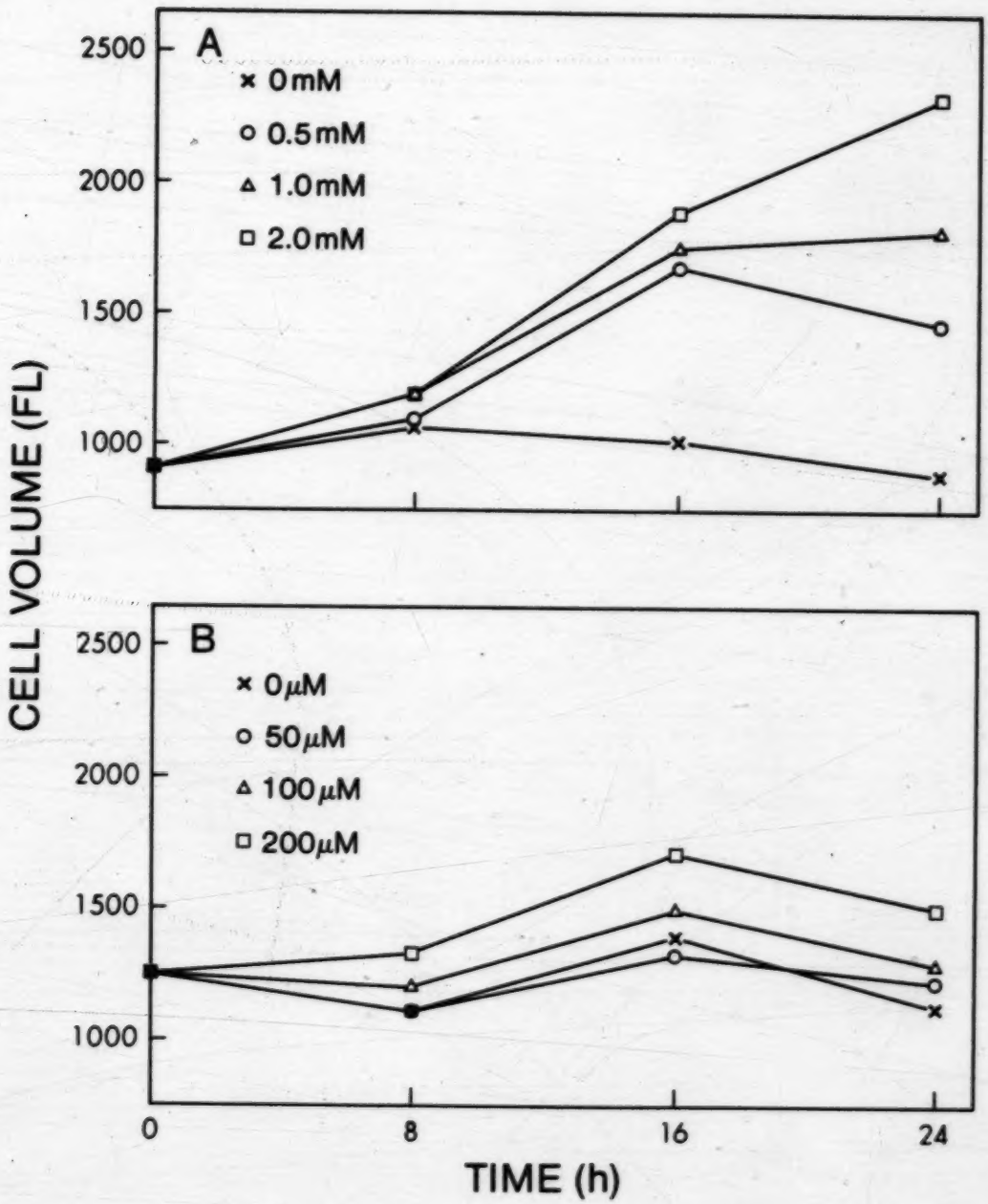



Fig. 3.2 Effect of IMPY (A) and INOX (B) on cell volume.

HU has been observed to cause an increase in cell volume of CHO cells in previous studies in this (Hordern, J. and Henderson, J.F., unpublished results) and in other laboratories [76] but in neither case were quantitative measurements made. Effects of 5HP on cell volume have been demonstrated using Sarcoma 180 ascites cells: a dose of 5HP which inhibited tumour cell growth in mice by 50% gave a 200% increase in the volume of these cells [56]. In contrast, cultured CHO-K1-C3 cells exposed to a 60% growth inhibitory concentration of 5HP only gave a 40% increase in modal cell volume. Increases in cell volume in the presence of 1.0 mM IMPY have been reported but cell volume was not quantitated [68]. With 1.0 mM IMPY, we observed a 60% increase in modal cell volume 24h after incubation. Plagemann *et al.* observed a 100% increase in cell volume of rat hepatoma cells exposed to a 100% growth inhibitory concentration of INOX (1.66 mM), and 75% and 25% increase in cell volume with *ca.* 75% (0.83 mM) and 50% (0.33 mM) growth inhibitory concentrations of INOX, respectively [76]. We found that a 55% growth inhibitory concentration gave a 49% increase in modal cell volume.

In this project, information regarding cell volume change are needed for the interpretation of nucleotide pool size data; this will be explained in the section dealing with pool sizes.

### 3.3 Cell Cycle.

The hypothesis that HU, 5HP, IMPY and INOX inhibit ribonucleotide reductase in intact cells logically seems to require that deoxyribonucleoside triphosphate pools decrease, leading to inhibition of DNA synthesis. Such biochemical changes should then be reflected in an altered progression of cells through the cell cycle, as such progression requires that DNA be synthesised. Therefore if synthesis of DNA is inhibited, cells should not proceed through the cycle in a normal fashion. Effects on the cell cycle are among the early biological reflections of biochemical changes in DNA synthesis in the cells; these effects usually can be observed before the cells have gone through one doubling, depending on which phase the cells are in and where the effects are. If only the synthesis of DNA is inhibited, cells in S phase will be affected and

this will cause an accumulation of cells at the $G_1$-S transition point.

To monitor the effects of the four drugs on the cell cycle, drugs were added to CHO cells and at 8h, 16h and 24h samples of cells were fixed, stained and analysed on the cell sorter as described in chapter 2. Control cultures to which saline had been added were run concurrently. The percentage of cells in the different phases of the cell cycle, *e.g.* $G_1$, S and $G_2M$, were analysed with the Coulter Electronics EASY I software (see Appendix for details). This computer program also arbitrary subdivides the S phase into four sections: early S, mid-$S_1$, mid-$S_2$ and late S.

Throughout the 24h incubation time, the control CHO cell population remained asynchronous (Fig. 3.3A, 3.5A, 3.7A and 3.9A), and this population of cells in log phase was therefore progressing through the cell cycle normally. The cell cycle results from drug-treated cells are presented in two ways: i) DNA distribution histograms, and ii) bar graphs of the number of cells in each phase expressed as a percentage of control.

**HU.** In the presence of 56 $\mu$M HU, no obvious synchronisation was observed (Fig. 3.3B), but there seemed to be more cells in S phase and fewer in $G_1$ phase. This observation was reflected in the cell cycle analysis results shown in Fig. 3.4 (expressed as a percentage of control); the percentage of cells in $G_1$ was lower than in controls while the number of cells in S phase was increased; this increase in S phase was mostly in early S and mid-$S_1$ phases. Similar but more dramatic effects on the cell cycle were observed with 100 $\mu$M and 200 $\mu$M HU (Fig. 3.3C and 3.3D); here, increases in S phase were not confined primarily to early S and mid-$S_1$ phases but were also observed in mid-$S_2$ and late S phases (Fig. 3.4). The distribution of cells within the S phase, especially for 100 $\mu$M and 200 $\mu$M doses, reflects the progression of synchronised cells through the S phase with time: at 8h, there were more cells in early S and mid-$S_1$ whereas by 24h, the percentage of cells in early S had dropped with a corresponding increase in mid-$S_2$ and late S phases.

**5HP.** A dose of 2.8 $\mu$M 5HP did not seem to have much effect on the progression of cells through the cell cycle (Fig. 3.5B and 3.6). However, the profiles of DNA histograms for

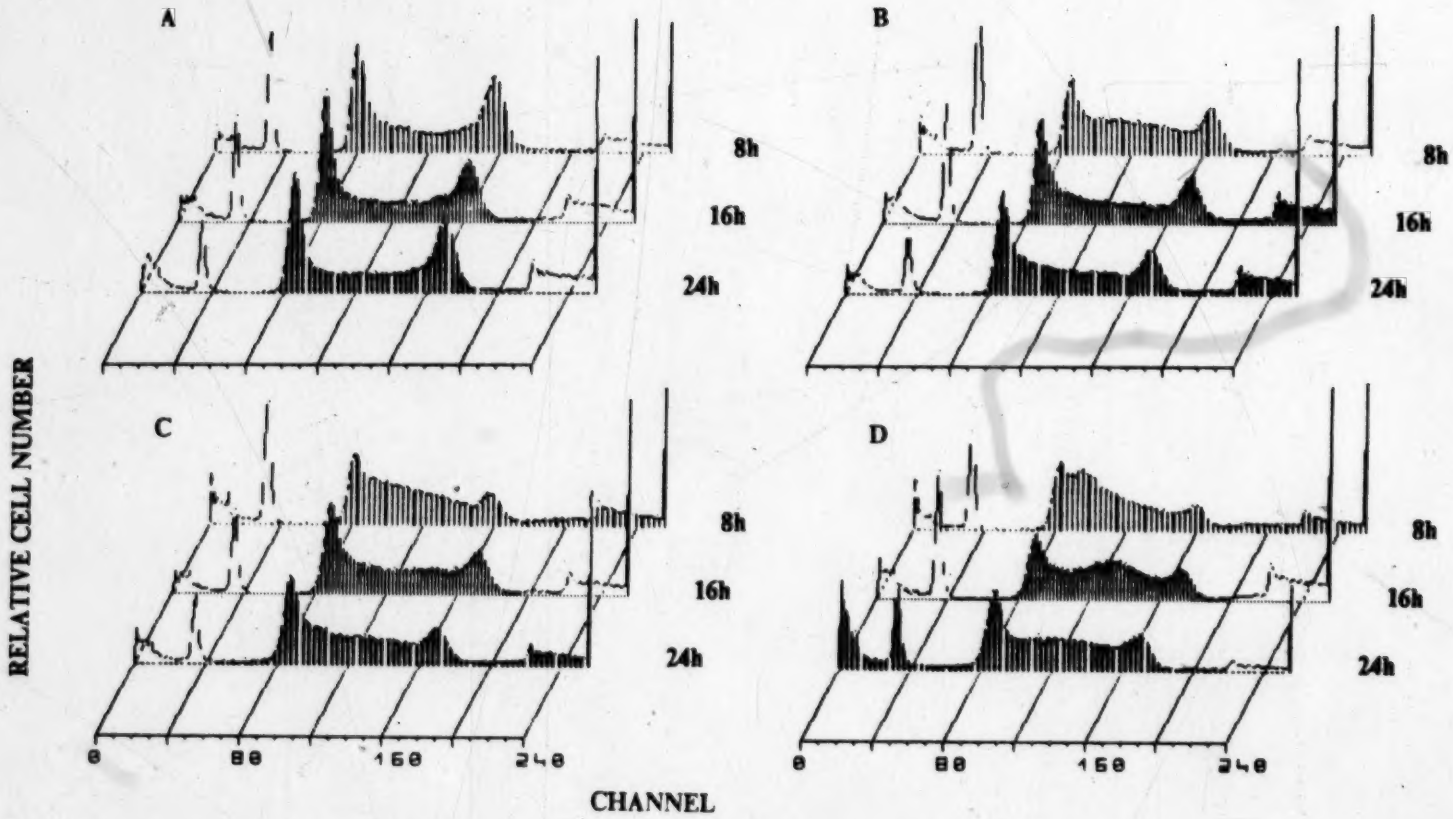



**Fig. 3.3 Effect of HU on the DNA distribution histograms of CHO cells.** Cells were treated for 8, 16, and 24h with 56 $\mu$M (B), 100 $\mu$M (C) and 200 $\mu$M (D) HU. A control distribution was determined at each time point and is shown in (A). Channel number is proportional to fluoresence intensity.

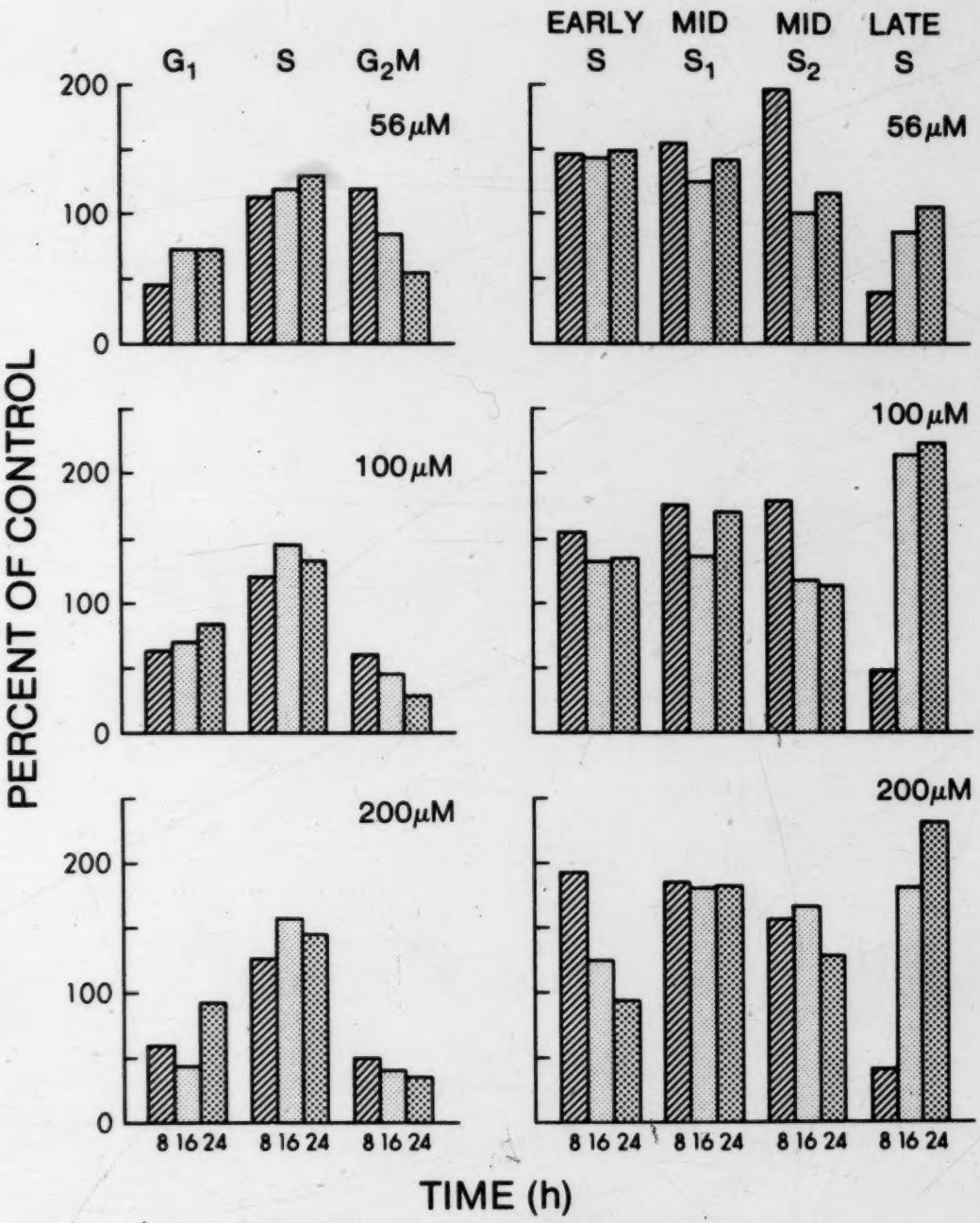



**Fig. 3.4 Relative distribution of HU-treated cells in different phases of cell cycle**

(Data recalculated from Fig. 3.3).

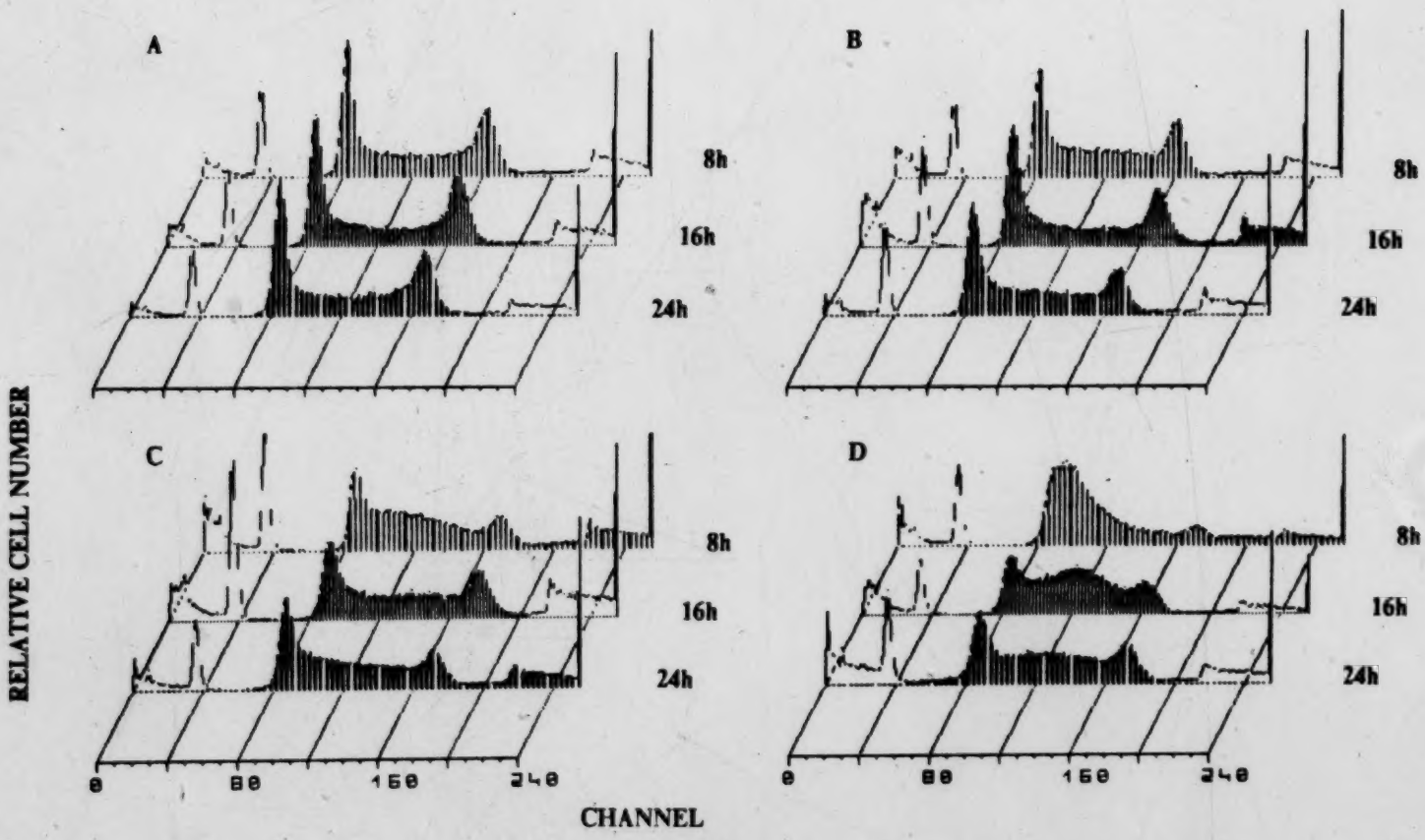



**Fig. 3.5 Effect of 5HP on the DNA distribution histograms of CHO cells.** Cells were treated for 8, 16, and 24h with 2.8 $\mu$M (B), 5.5 $\mu$M (C) and 10.0 $\mu$M (D) 5HP. A control distribution was determined at each time point and is shown in (A). Channel number is proportional to fluoresence intensity.

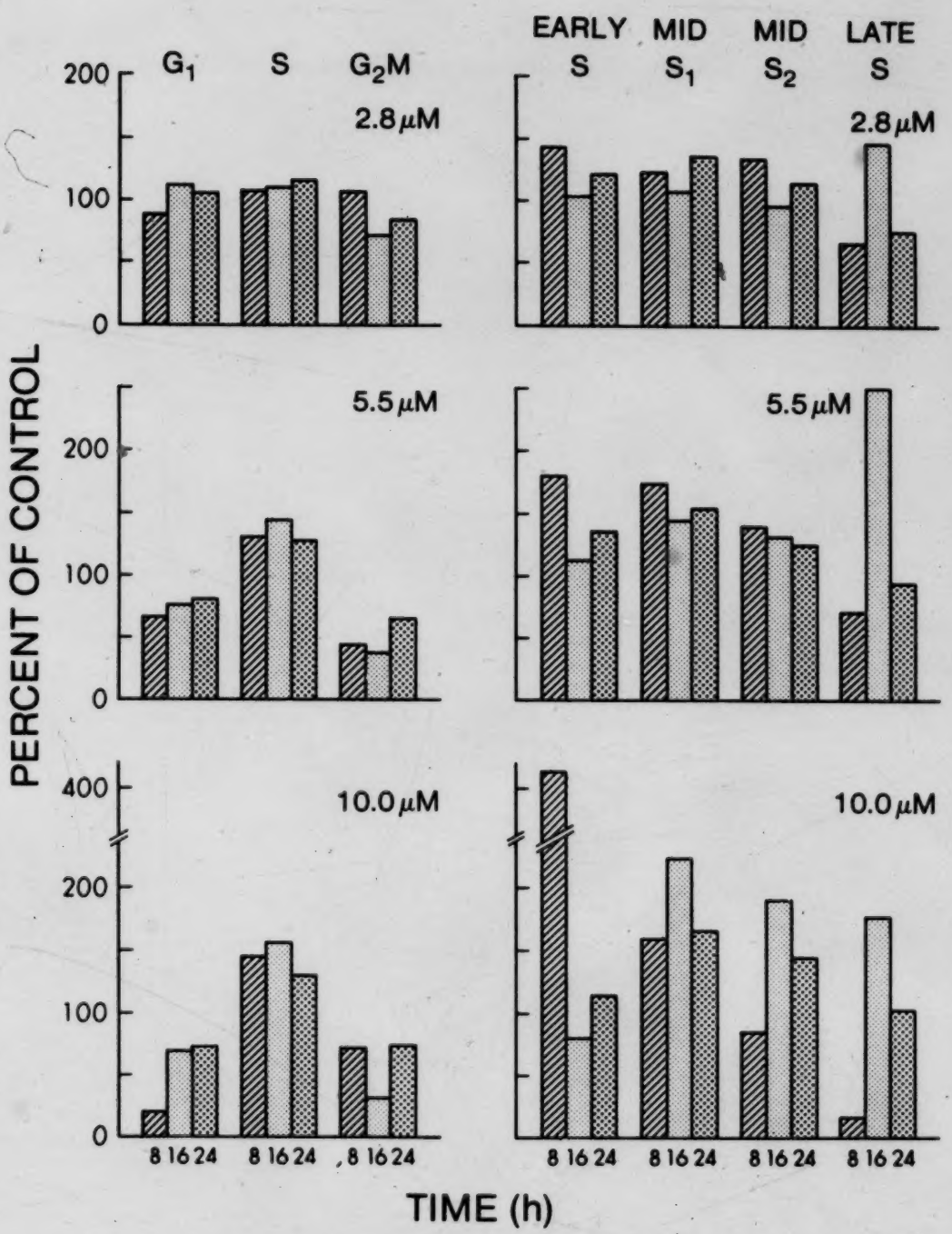



**Fig. 3.6 Relative distribution of 5HP-treated cells in different phases of cell cycle** (Data recalculated from Fig. 3.5).

5.5 $\mu$M and 10.0 $\mu$M 5HP were similar to those of HU (Fig. 3.5C and 3.5D), and there were more cells in S phase than in $G_1$ or $G_2M$ phases, relative to control cultures. At 8h, CHO cells exposed to 10 $\mu$M 5HP accumulated at early S-mid $S_1$ phases (Fig. 3.6) and 8 hours later most of the accumulated cells had started to progress through the S phase; by 24h the DNA histogram profile was begining to resemble that of the unperturbed control cells (Fig. 3.5A).

**IMPY.** The concentrations of IMPY used in these experiments seemed to have a more potent effect on the cell cycle. The effects of the lowest concentration of IMPY , 0.5 mM, were similar to that of 5.5 $\mu$M 5HP or of 100 $\mu$M HU. The progression of synchronised cells through the different stages of S phase with time was most evident with 1.0 mM and 2.0 mM IMPY (Fig. 3.7C and 3.8). With 2.0 mM IMPY, CHO cells were found to accumulate in $G_1$ phase at 8h, and by 16h most of these cells had entered S phase (Fig.3.7D).

**INOX.** INOX had a different effect on the cell cycle, as CHO cells exposed to 200 $\mu$M INOX appeared to accumulate at the $G_2M$ phase (Fig. 3.9D and 3.10). Concentrations of 50 $\mu$M and 100 $\mu$M did not seem to have any effect on the progression of cells through the cycle.

From our results with HU-treated cells, we can conclude that the progression of cells through S phase is affected. These cells tend to accumulate in early S phase rather than in $G_1$ (Fig. 3.4); similar observations were made with 5HP. With IMPY, cells accumulated in $G_1$ as well as in S while cells in $G_2M$ proceeded normally (Fig. 3.5 and 3.5). In contrast, INOX-treated cells were able to proceed through the S phase in an apparently normal fashion but cells in $G_2M$ were prevented from progressing (Fig. 3.9D).

Accumulation of cells in the S phase indicates that synthesis of DNA is specifically inhibited. These results, though not conclusive, are consistent with the hypothesis that ribonucleotide reductase in intact cells is inhibited by HU, 5HP and IMPY.

In the case of INOX, accumulation of cells at $G_2M$ and apparent normal progression through S phase suggests that the synthesis of DNA is unaffected or only mildly affected. Therefore, under our experimental conditions, INOX does not seem to inhibit ribonucleotide reductase in intact cells.

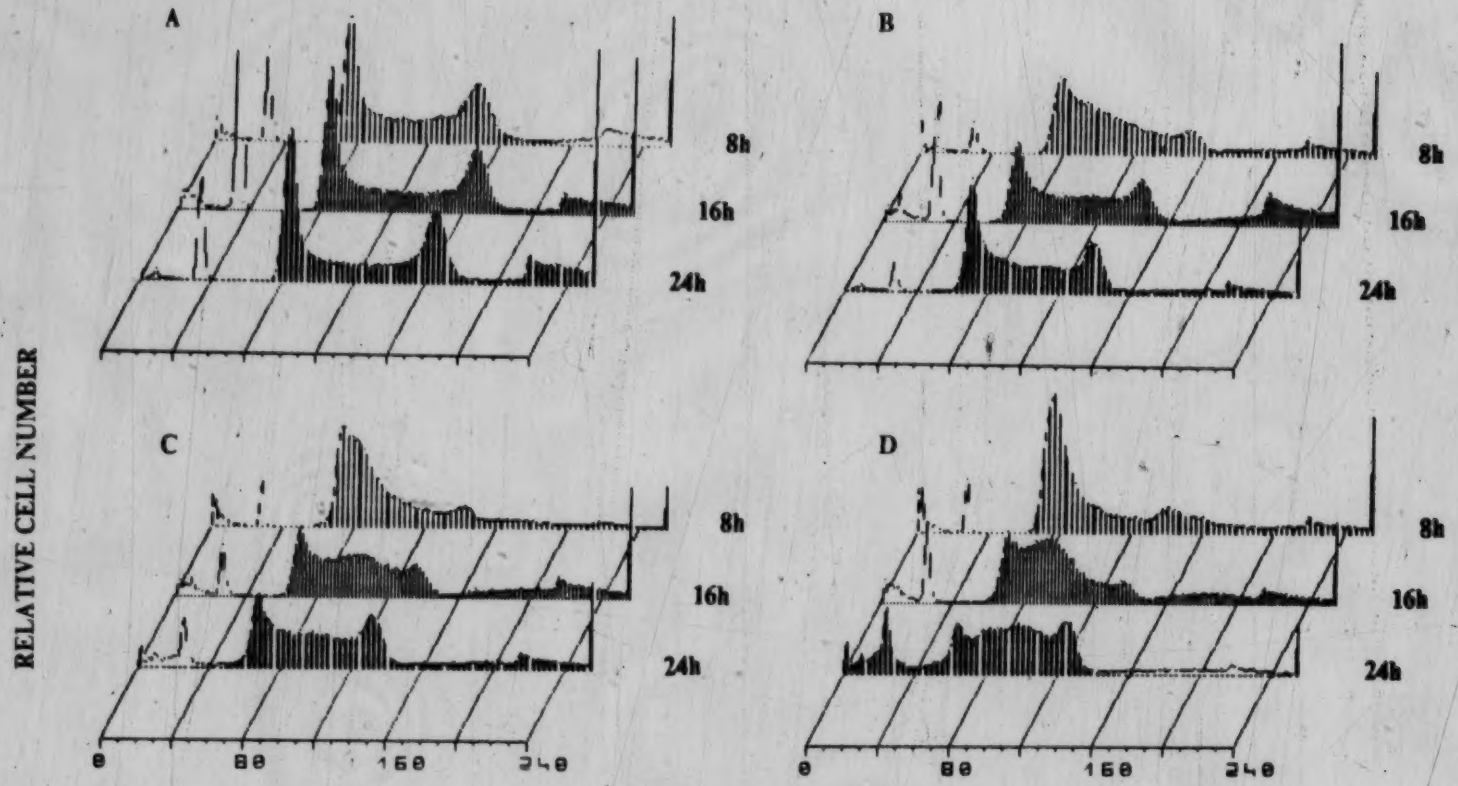



**Fig. 3.7 Effect of IMPY on the DNA distribution histograms of CHO cells.** Cells were treated for 8, 16 and 24h with 0.5 mM (B), 1.0 mM (C) and 2.0 mM (D) IMPY. A control distribution was determined at each time point and is shown in (A). Channel number is proportional to fluoresence intensity.

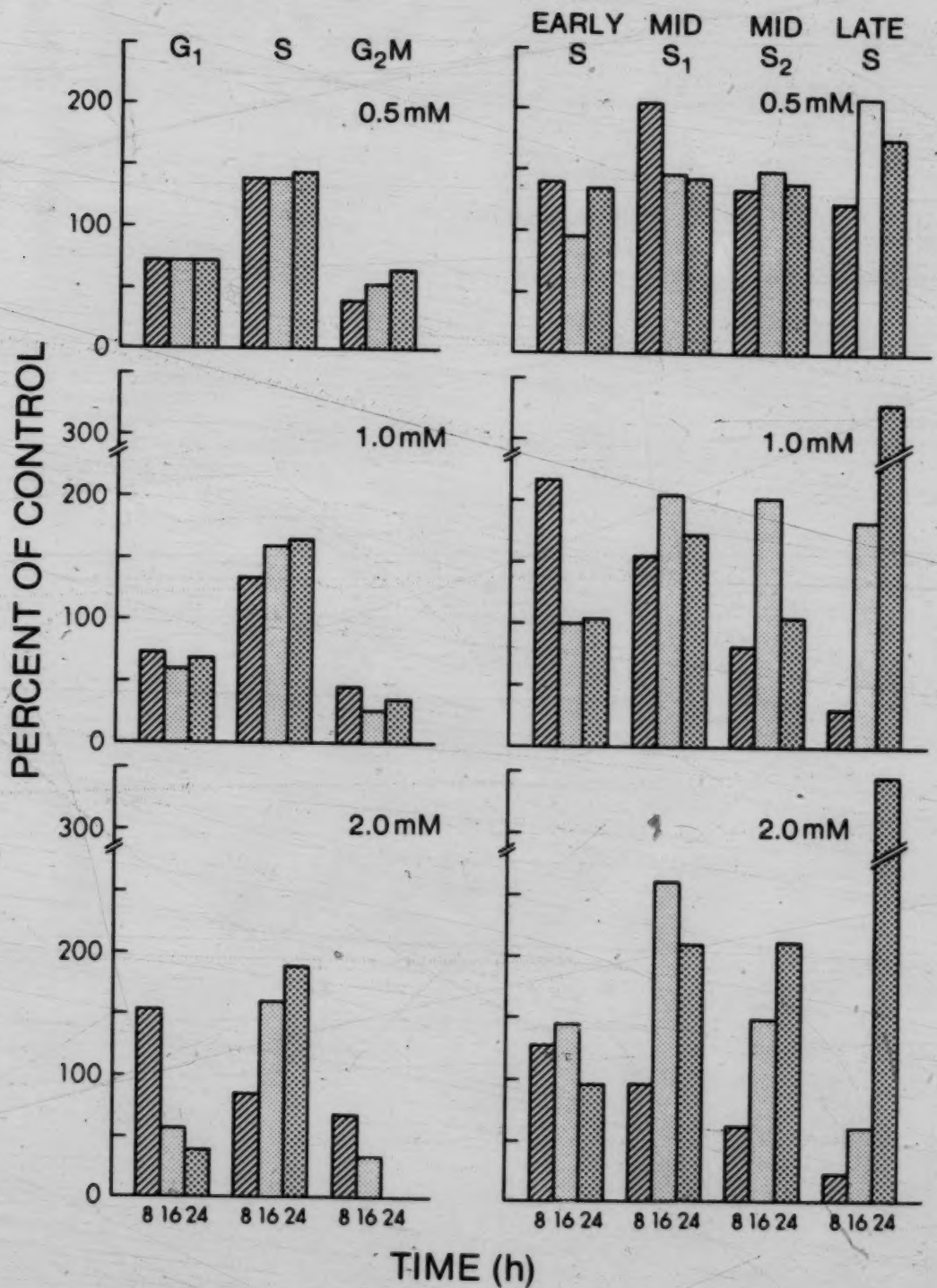



**Fig. 3.8 Relative distribution of IMPY-treated cells in different phases of cell cycle** (Data recalculated from Fig 3.7).

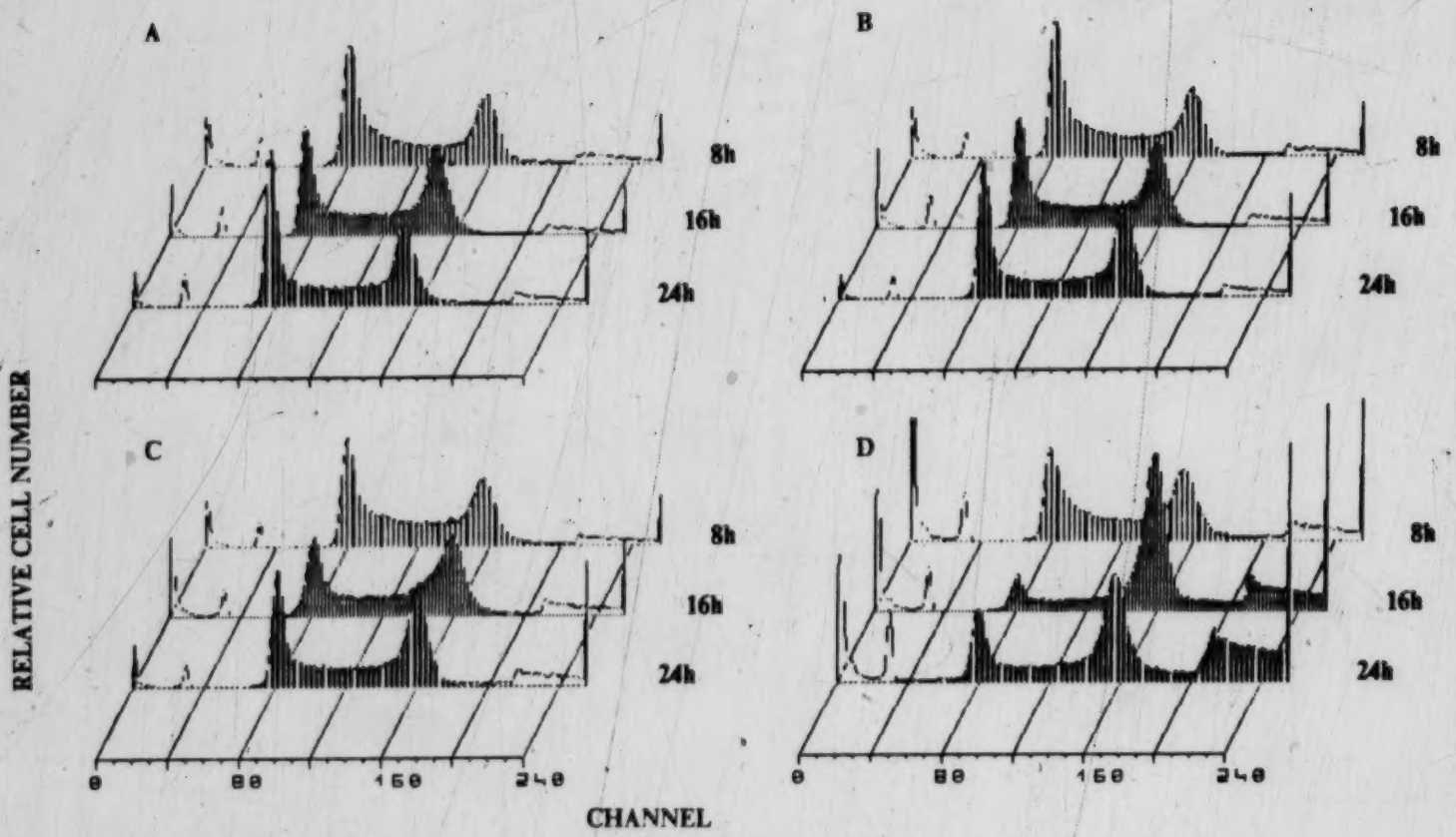



**Fig. 3.9 Effect of INOX on the DNA distribution histograms of CHO cells.** Cells were treated at 8, 16 and 24h with 50 $\mu$M (B), 100 $\mu$M (C) and 200 $\mu$M (D) INOX. A control distribution was determined at each time point and is shown in (A). Channel number is proportional to fluoresence intensity.

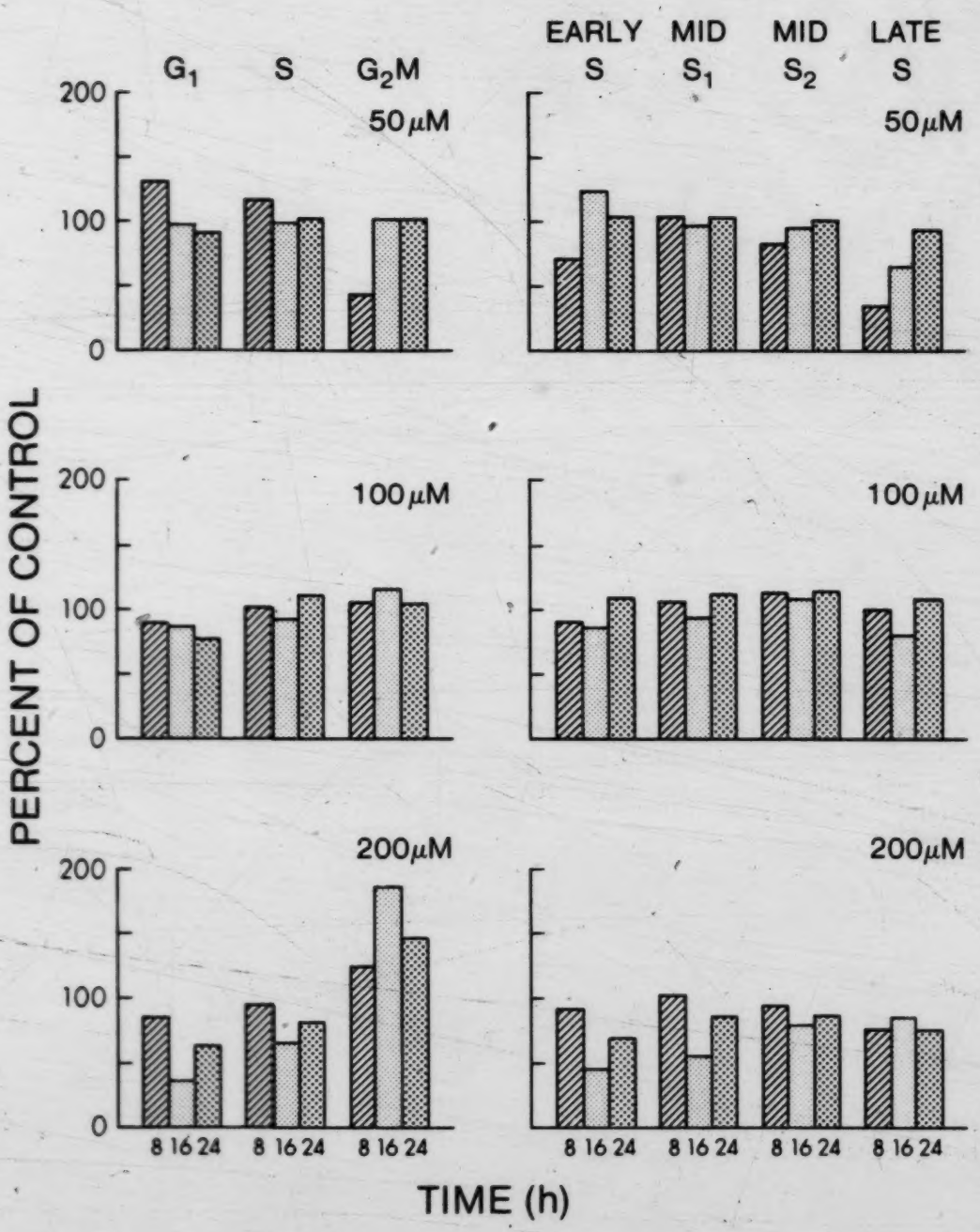



**Fig. 3.10 Relative distribution of INOX-treated cells in different phases of cell cycle** (Data recalculated from Fig. 3.9).

Adams and Lindsay [40] showed that incubation of L cells with 2 mM HU for 18h resulted in synchronised DNA synthesis, and cell cycle analysis of Novikoff cells treated with 1 mM HU for 6h also showed that a large percentage of cells was blocked in $G_1$ and early S phases [76]; there were very few cells in S and the $G_2M$ peak was not visible. We did not observe such dramatic effects with CHO cells, but much lower concentrations were used. However, we did obtain synchronisation of CHO cells in S phase, but this block was incomplete and hence a proportion population of cells progressed through S phase with time. Bhuyan *et al.* [57] demonstrated that 50 $\mu$M 5HP was most cytotoxic when added to DON cells in S phase. Consistent with this, we observed that 5HP caused CHO cells to synchronise in S phase. P388 and L1210 cells in mice were shown to synchronise in S phase after treatment with IMPY [70]. Our DNA distribution histogram of IMPY-treated cells were similar to those obtained using human lymphoblasts [69]: treatment with 0.5 mM, 1.0 mM and 2.0 mM concentrations of IMPY for two 18h period separated by a 10h release period, resulted in accumulation of cells in $G_1$ and in early S. After 6h incubation with 0.83 mM INOX, a large proportion of Novikoff cells were arrested in $G_2M$ phase [76]; the $G_1$ peak had disappeared completely; this is consistent with our data with CHO cells.

### 3.4 Ribonucleotide and Deoxyribonucleotide Concentrations.

Drugs that are specific for ribonucleotide reductase should reduce the concentration of one or more deoxyribonucleotide triphosphates. In order to test our hypothesis, we therefore measured deoxyribonucleoside triphosphate levels of CHO cells exposed to each drug. CHO cells in log-phase were incubated with the appropriate drugs, and at 8h, 16h, and 24h time points, acid soluble fractions were prepared. Control cultures to which saline was added were run concurrently. Deoxyribonucleoside triphosphate levels were determined by the polymerase assay and ribonucleotide levels were determined using HPLC (as described in Chapter 2).

As shown in a preceeding section, modal cell volumes sometimes also increased at the same points, and the results obtained from the cell volume experiments were therefore used as

correction factors for the pool sizes. This was done because pool size results are usually expressed on a per cell basis, assuming a constant modal cell volume throughout the experiment. Assuming that intracellular contents are proportional to cell volume, correcting for cell volume changes will eliminate this additional complication in the analysis of pool size data; therefore, any pool size change observed will be due solely to the effect of drugs. Both the uncorrected and corrected pool size data are presented as a percentage of control values in Fig. 3.11 - Fig. 3.18. The control values are 5.97, 0.98, 0.56 and 1.90 nmoles per $10^6$ cells for ATP, GTP, CTP, and UTP, respectively, and 20.9, 9.7, 52.4 and 57.0 pmoles per $10^6$ cells for dATP, dGTP, dCTP and dTTP, respectively.

In general, all four ribonucleotides increased with time in the uncorrected data, especially with the highest concentrations of drugs. But, after taking into account the cell volume changes, there was little or no increase in ribonucleotide pools (Fig. 3.11- Fig. 3.17), except for INOX-treated cells where ribonucleotide pool sizes were still elevated after correction (Fig. 3.17 - Fig. 3.18).

**HU.** The effects of HU on ribonucleotide and deoxyribonucleotide pools are shown in Fig. 3.11 and Fig. 3.12. With respect to uncorrected deoxyribonucleotide concentrations, dATP levels were decreased, dCTP pools showed the greatest increases, and dGTP and dTTP pools also showed increases at 16h and 24h for all three drug concentrations (Fig. 3.11). After considering cell volume effects, dTTP and dGTP pools did not appear to be much affected by HU (Fig. 3.12); however, dATP levels consistently remained below 50% of control values. The increase in dCTP was still present but to a lesser degree; dCTP pools were highest at 16h, whilst at 24h the large cell volume correction factor meant that in fact there was a less than 50% increase in pool size. After accounting for cell volume effects, the largest dCTP pool increases was observed with cells exposed to 56 $\mu$M HU for 16h and the smallest dATP pool size was associated with an 8h incubation with 200 $\mu$M HU.

**5HP.** The general pattern of response of deoxyribonucleotides to 5HP was similar to that of HU, in both corrected and uncorrected data (Fig. 3.13 - Fig. 3.14): an overall decrease

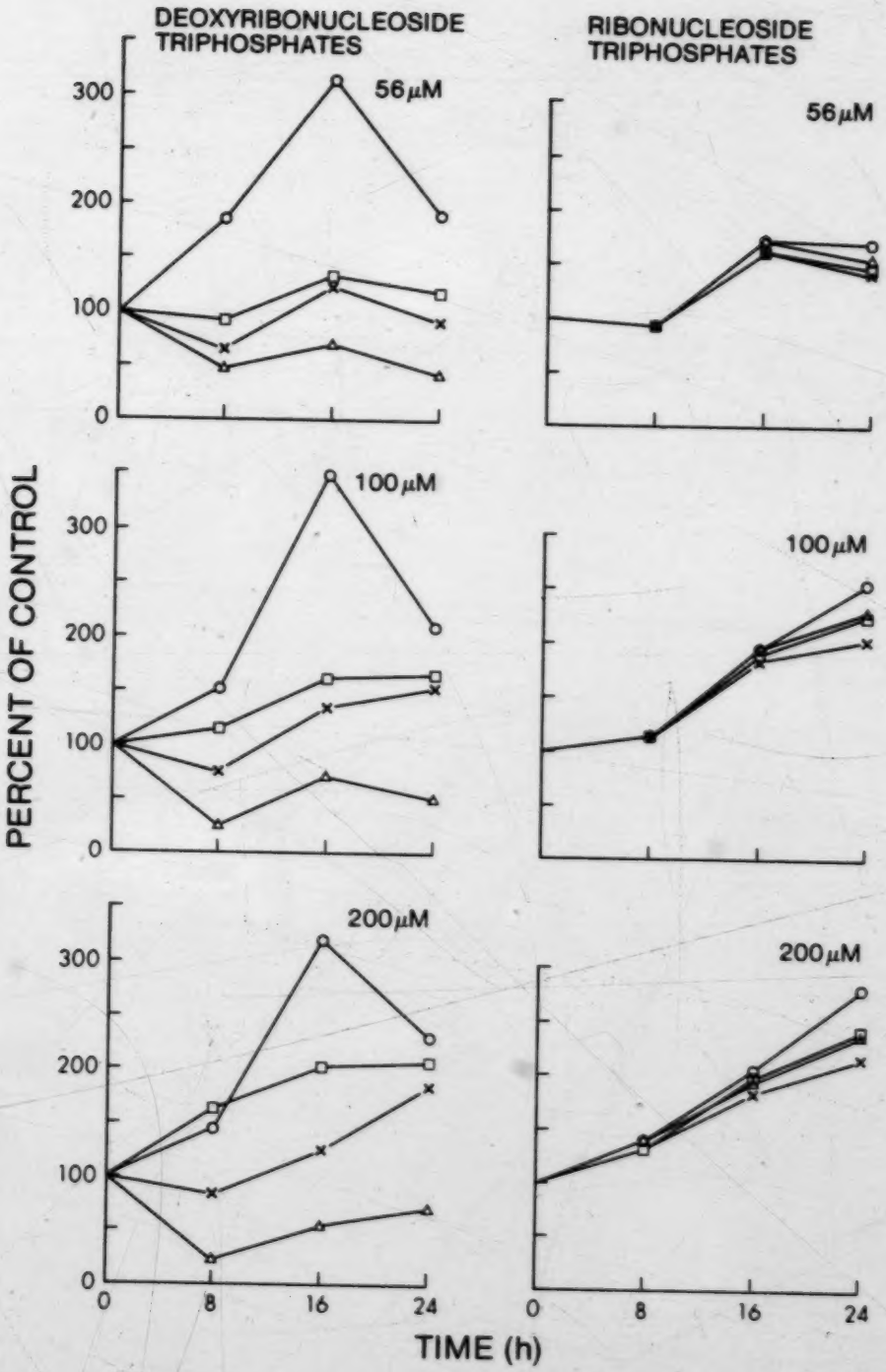



**Fig. 3.11** Effect of HU on deoxyribonucleotide and ribonucleotide concentrations (△,dATP/ATP; □,dGTP/GTP; ○,dCTP/CTP; x,dTTP/UTP).

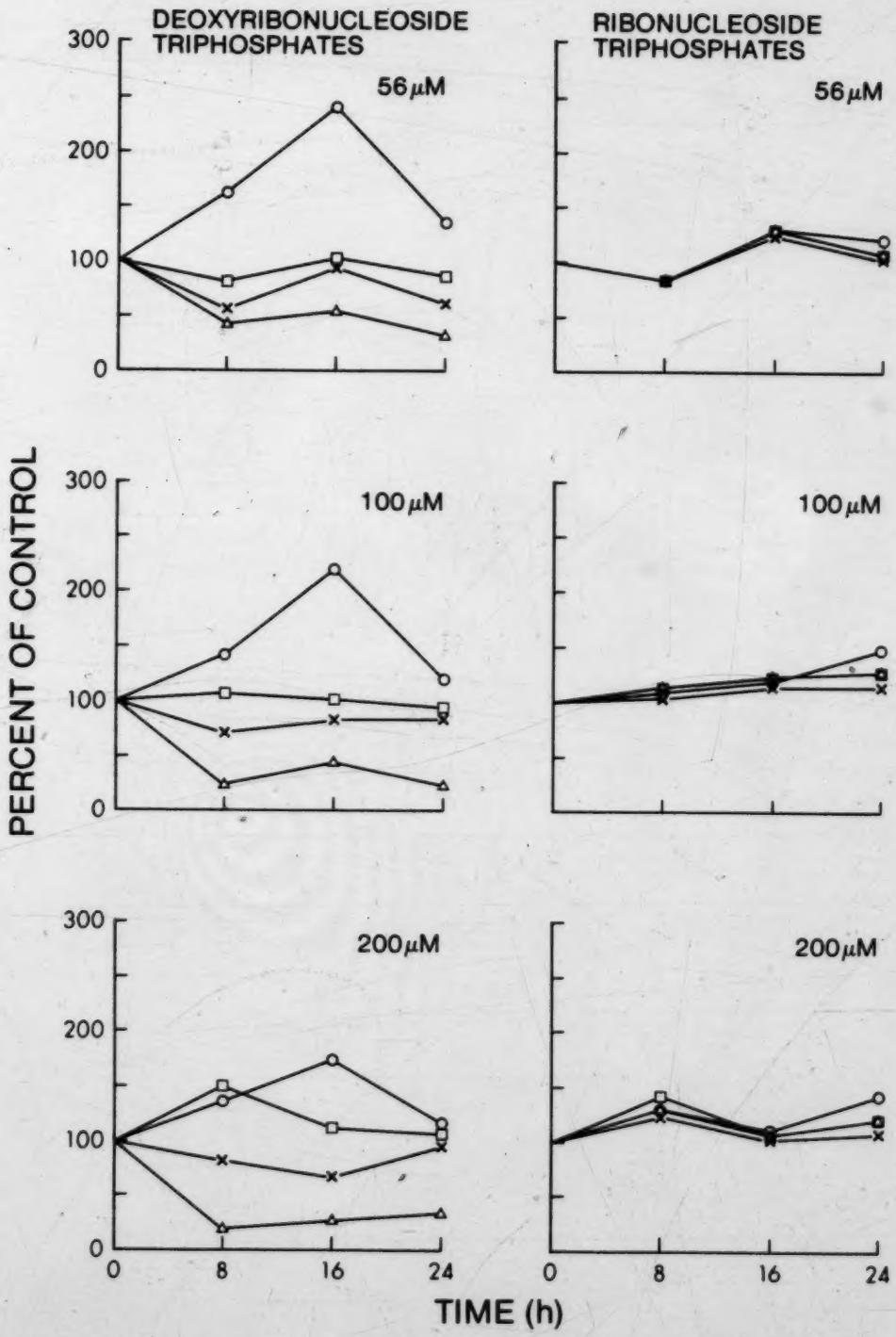



**Fig. 3.12** Deoxyribonucleotide and ribonucleotide concentrations of HU-treated cells corrected for cell volume changes. ( △ ,dATP/ATP; □ ,dGTP/GTP; ○ ,dCTP/CTP; x ,dTTP/UTP).

in dATP and increase in dCTP were the main findings in both corrected and uncorrected data. Small decreases in dTTP and dGTP were observed at 8h followed by an increase at 24h for both 2.8 $\mu$M and 5.5 $\mu$M 5HP in the uncorrected data, but in the case of the highest concentration, dGTP and dTTP increased consistently with time (Fig. 3.13). After correcting for cell volume changes, dTTP and dGTP levels in cells exposed to 2.8 $\mu$M and 5.5 $\mu$M 5HP were observed to be between 60% and 100% of control (Fig. 3.14). As with 200 $\mu$M HU, dGTP was higher than dCTP at 8h at the highest dose of 5HP.

**IMPY.** With IMPY, dATP pools sizes were decreased at all three drug concentrations, dCTP levels were elevated in cells exposed to 0.5 and 1.0 mM IMPY in both corrected and uncorrected data (Fig. 3.15 - 3.16). In general, deoxyribonucleotide pools were highest at 24h in the uncorrected data and at 8h in the corrected data. CHO cells exposed to 0.5 mM and 1.0 mM IMPY had large dCTP pools in the corrected data but with 2 mM IMPY, dGTP levels were even the higher. The response of the dTTP pool to IMPY was similar to that of HU and 5HP-treated cells.

**INOX.** Unlike the effects of HU, 5HP and IMPY, correcting the data from INOX-treated cells for cell volume changes produced no major differences in pool sizes because of the relatively small increases in cell volume; therefore, no comparison between corrected and uncorrected pool sizes will be made although both sets of data are shown (Fig. 3.17 - 3.18). INOX appears to have very little effect on deoxyribonucleotide pools at these concentration ranges.

The main aim of these experiments was to test our hypothesis that these drugs inhibit ribonucleotide reductase in intact cells. This hypothesis includes the assumption that the phosphorylation of deoxyribonucleoside diphosphates to triphosphates and subsequent incorporation into DNA are not directly affected by these drugs. Deoxyribonucleoside triphosphates were measured instead of diphosphates because triphosphates concentrations are more easily and more accurately measured.

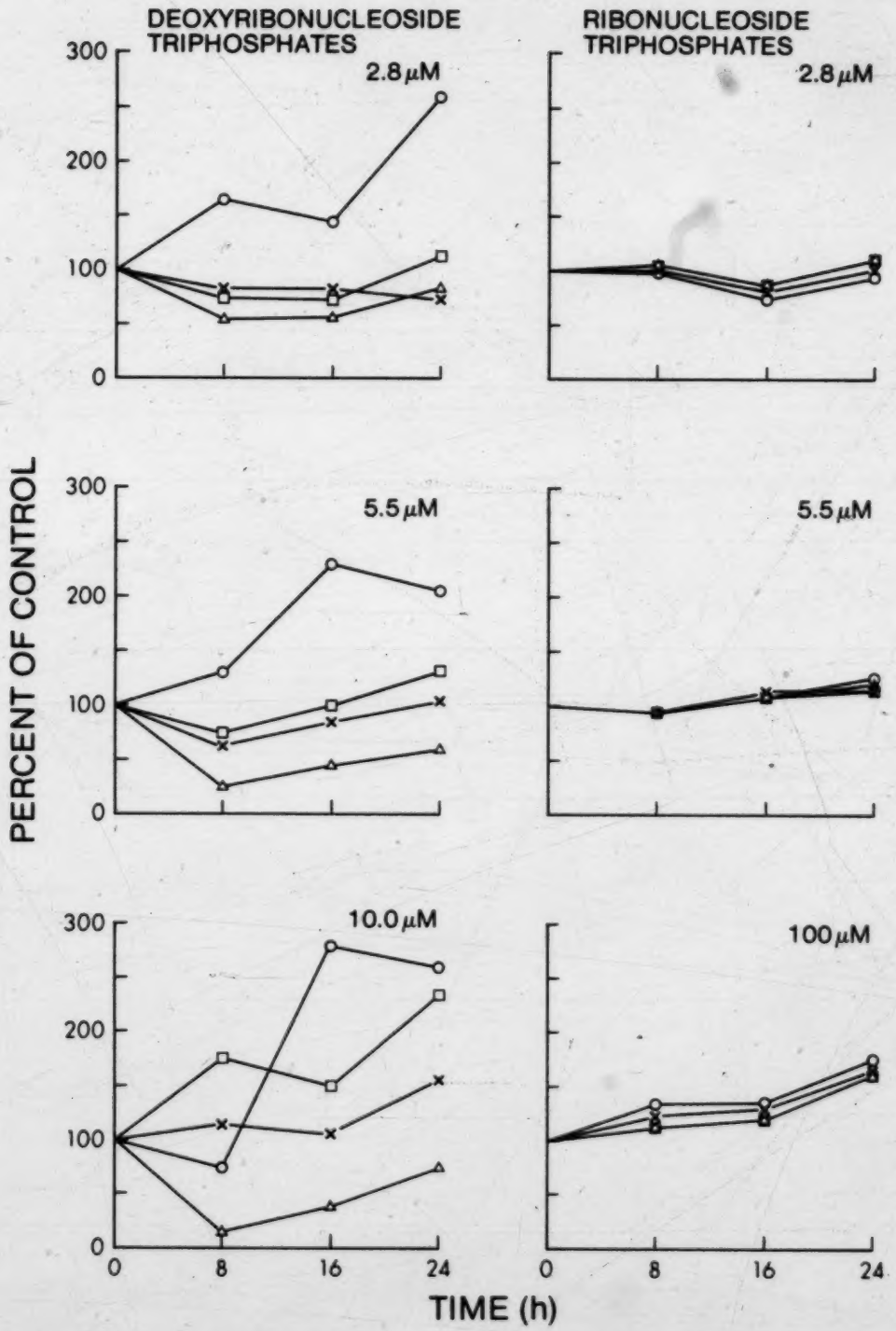



**Fig. 3.13 Effect of 5HP on deoxyribonucleotide and ribonucleotide concentrations**

**( △ ,dATP/ATP; □ ,dGTP/GTP; ○ ,dCTP/CTP; x ,dTTP/UTP).**

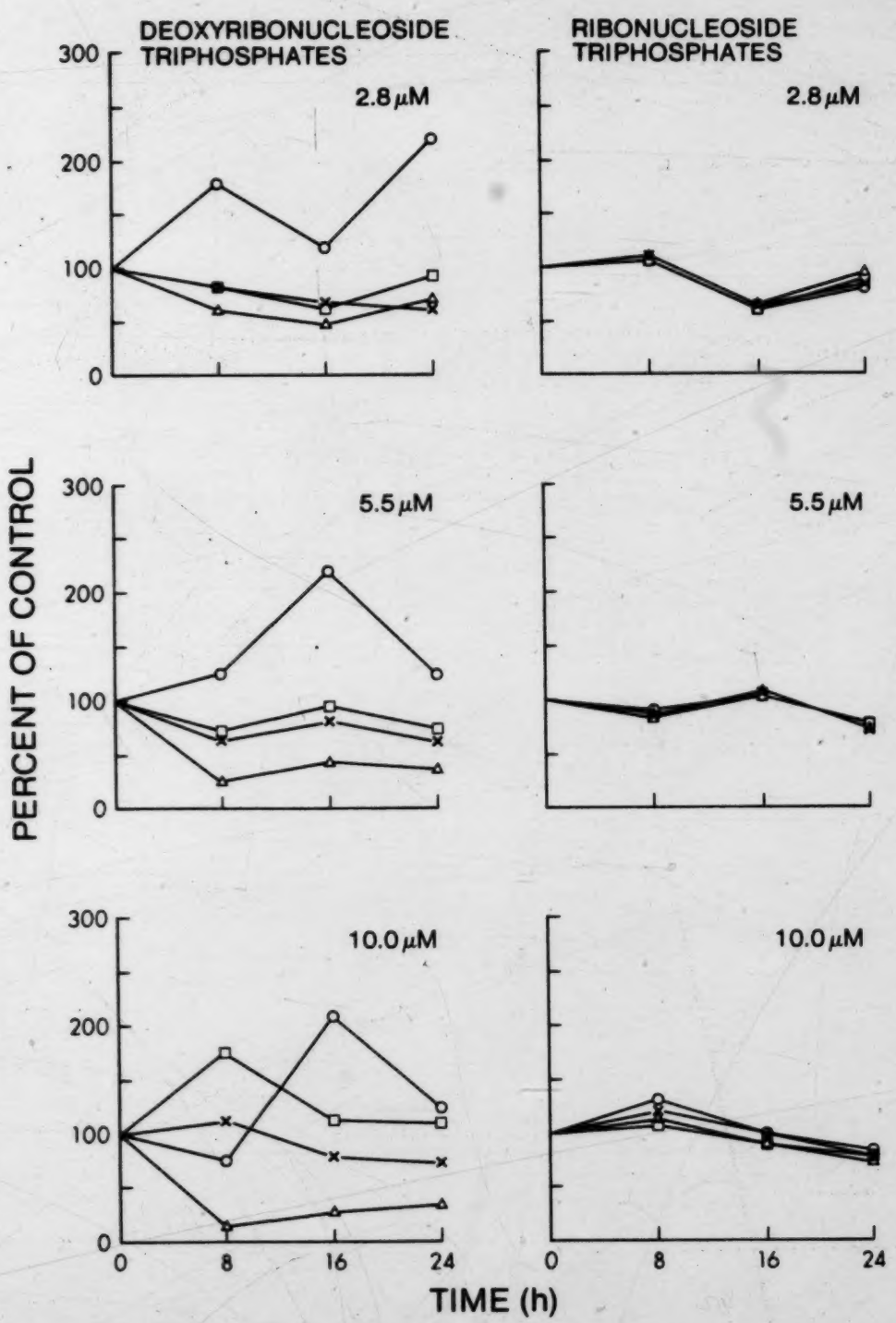



**Fig. 3.14 Deoxyribonucleotide and ribonucleotide concentrations of 5HP-treated cells corrected for cell volume changes (△ ,dATP/ATP; □ ,dGTP/GTP; ○ ,dCTP/CTP; x ,dTTP/UTP).**

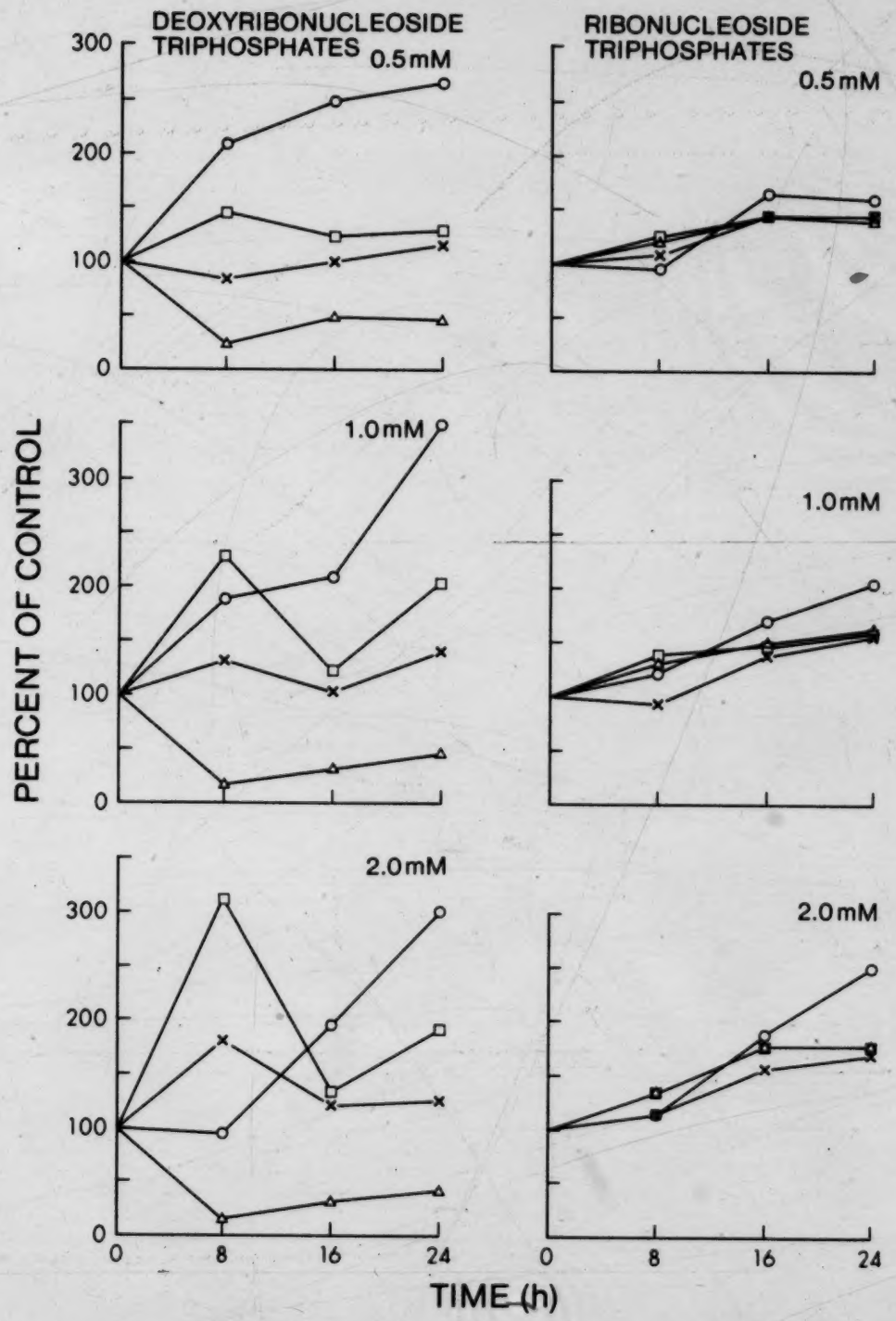



**Fig. 3.15 Effect of IMPY on deoxyribonucleotide ribonucleotide concentrations**

**( △ ,dATP/ATP; □ ,dGTP/GTP; ○ ,dCTP/CTP; x ,dTTP/UTP).**

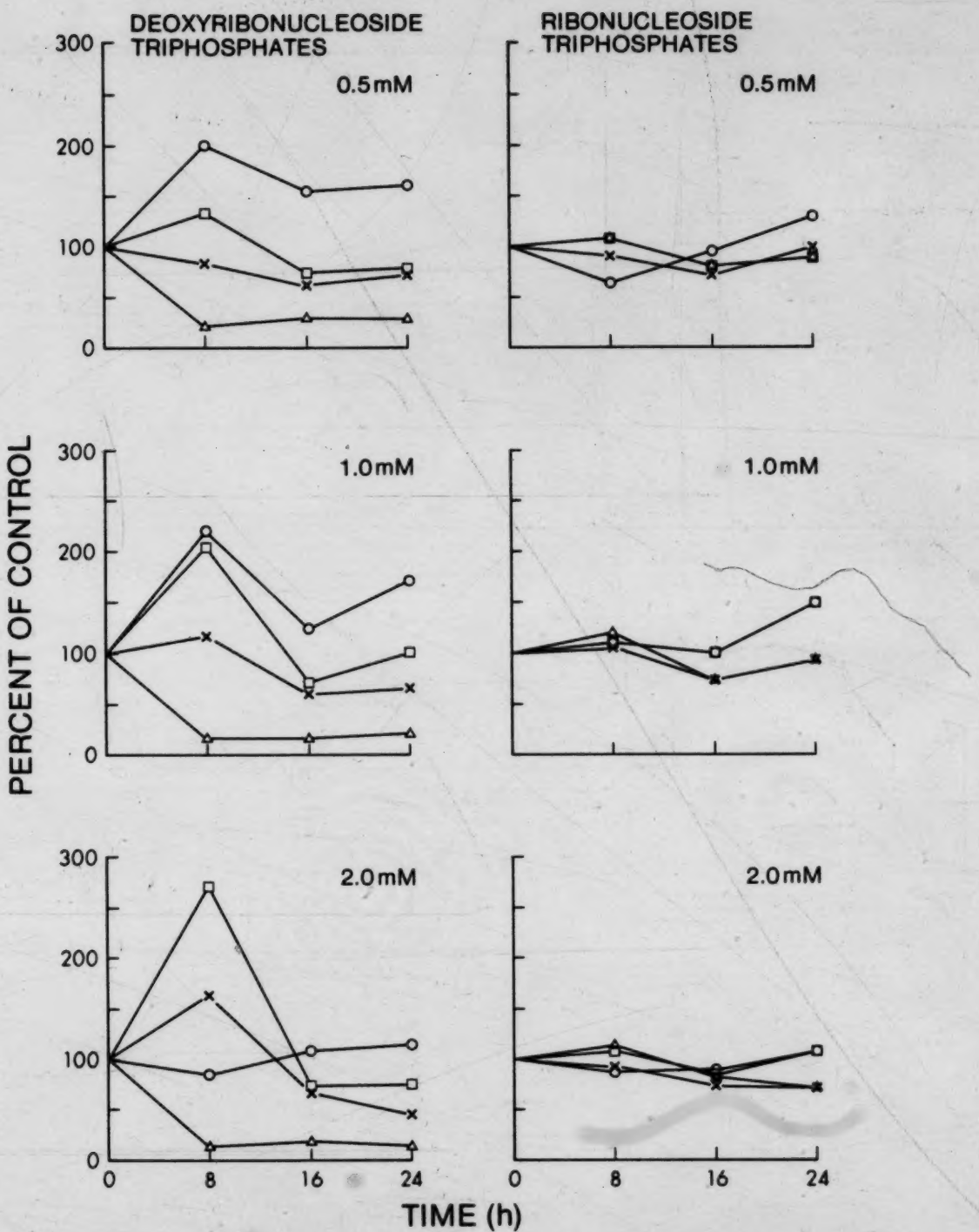



**Fig. 3.16** Deoxyribonucleotide and ribonucleotide concentration of IMPY-treated cells corrected for cell volume changes ( △ ,dATP/ATP; □ ,dGTP/GTP; ○ ,dCTP/CTP; × ,dTTP/UTP).

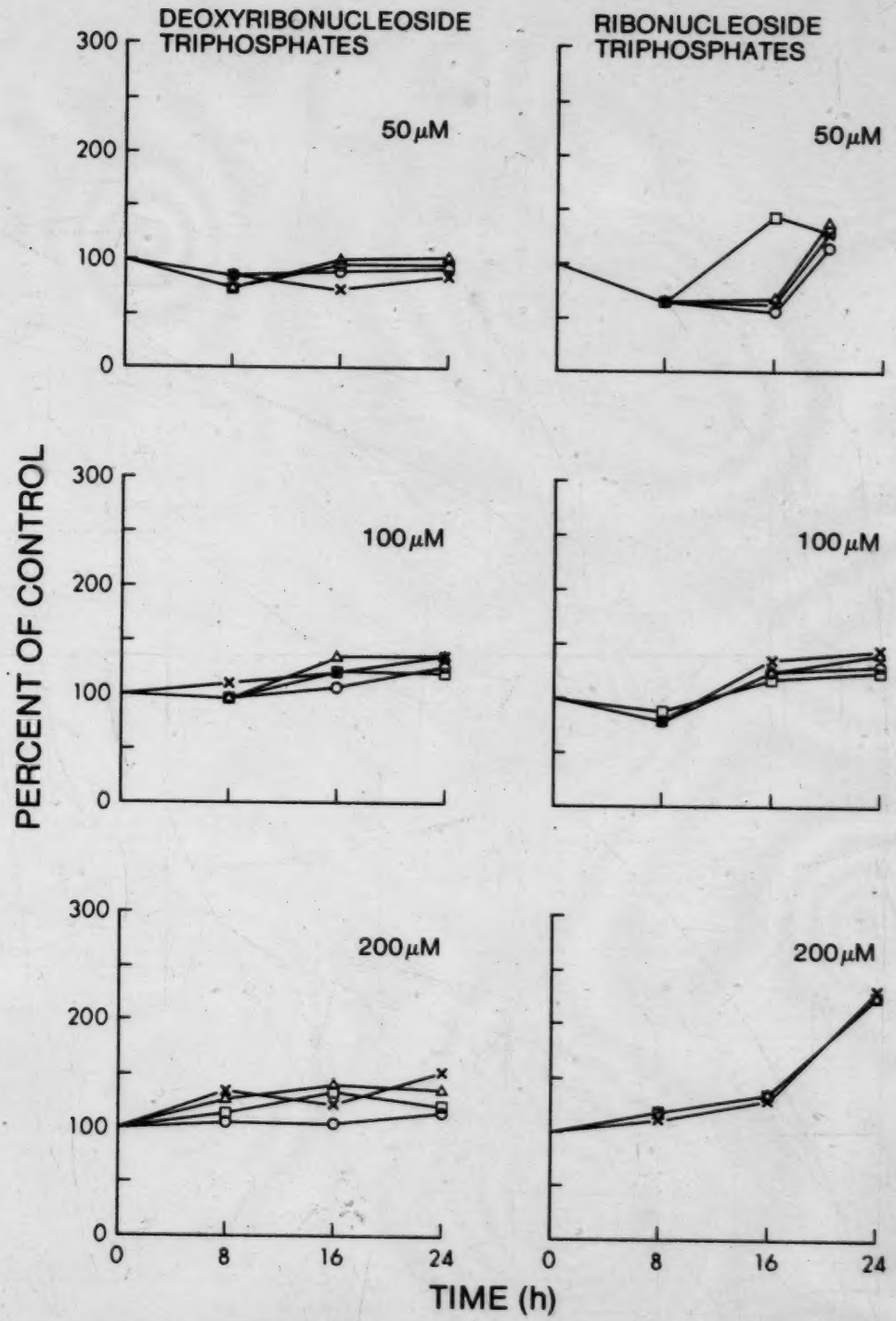



**Fig. 3.17 Effect of INOX on deoxyribonucleotide and ribonucleotide concentrations** ( △ ,dATP/ATP; □ ,dGTP/GTP; ○ ,dCTP/CTP; × dTTP/UTP).

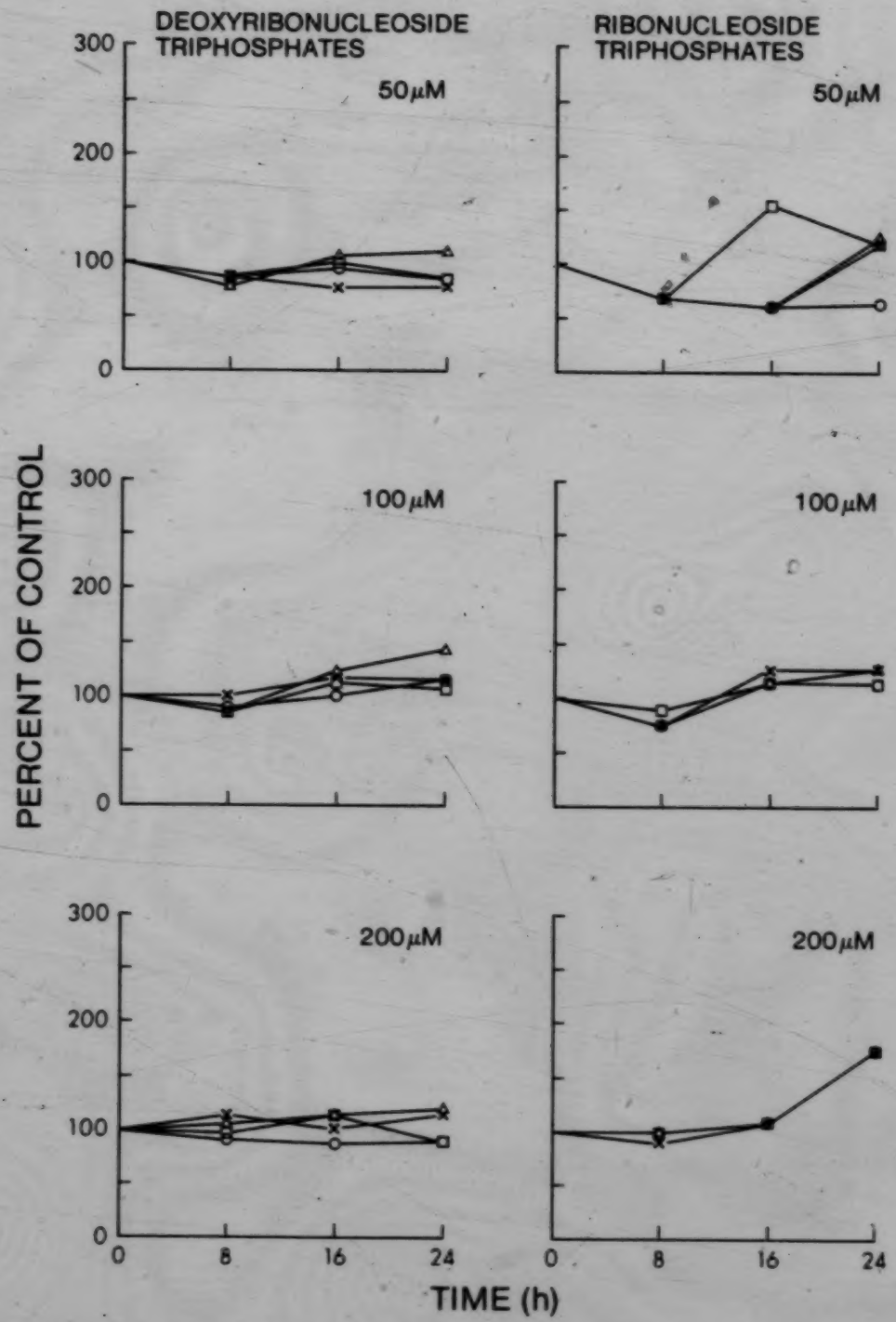



**Fig. 3.18** Deoxyribonucleotide and ribonucleotide concentrations of INOX-treated cells corrected for cell volume changes ( △ ,dATP/ATP; □ ,dGTP/GTP; ○ ,dCTP/CTP; x ,dTTP/UTP).

If the hypothesis is correct, then the most straightforward result of these experiments would be a decrease in all four deoxyribonucleoside triphosphates. However, we did not observe this in our experiments and hence the proposed hypothesis does not appear to be correct for the four drugs tested. If all four deoxyribonucleotides did decrease, then we could infer that all four ribonucleoside diphosphates may share a common active site on ribonucleotide reductase; therefore, based on this prediction, our results imply that the hypothesis of four substrates sharing one active site may not be correct.

It is possible, however, that the hypothesis is still correct, but that the changes observed in deoxyribonucleoside triphosphate concentrations in drug-treated cells are the result of a combination of incomplete inhibition of ribonucleotide reductase and complex regulation of the enzyme. Then the initial incomplete inhibition of ribonucleotide reductase would lead to a decrease in all four deoxyribonucleotides, and each decreased deoxyribonucleotide, in turn, would have its own allosteric effect on ribonucleotide reductase. For example, based on Thelander and Reichard's model [1] and Hunting and Henderson's work using CHO cells [5,79], a decrease in dATP concentration in cells would result in an increase in the other three deoxyribonucleotides, a decrease in dGTP concentrations would cause dCTP levels to increase but dATP levels to decrease, a decrease in the dTTP pool would result in increased dCTP concentrations and decreased dGTP levels, and lastly, a decreased dCTP level would have no effect on the reduction of the other three ribonucleotides. The overall effect here, could be no change in dGTP levels, decreased dATP levels, elevated dTTP concentrations and greatly elevated dCTP levels. This conclusion is based on the assumption that all four ribonucleoside diphosphate reduction processes are inhibited to the same extent simultaneously, and that resultant secondary changes are of similar magnitude.

If the enzyme is as highly regulated as reputed, then these secondary changes in deoxyribonucleotide pools would have further feedback effects: decreased dATP concentrations would lead to elevation of the other three deoxyribonucleotides, increased dCTP would have no effect, and increased dTTP would cause dGTP levels to increase but dCTP levels to decrease.

The overall outcome could be decreased dATP, a small increase in dGTP, moderately increased dTTP and great increases in dCTP.

**HU.** Our results with HU agree with the predictions just described, in that dCTP levels were increased and dATP levels were decreased (Fig. 3.12). But they disagree in that there was no increase in dTTP, and dGTP concentrations were usually close to control values in HU-treated cells except with 200 $\mu$M HU; in that case dGTP levels were increased by 50% at 8h. Therefore, based on the predictions stated above, our results with HU suggests that the hypothesis is not correct for HU.

**5HP.** Like HU-treated cells and in agreement with the predictions just described, dCTP levels increased and dATP levels decreased in 5HP-treated cells (Fig. 3.14). However, these results disagree with the predictions in that neither dTTP nor dGTP concentrations increased in cells, except at 8h with 10 $\mu$M 5HP, where an increase in dGTP levels was observed. As the results are not completely consistent with the predictions, this implies that the hypothesis is not correct for 5HP.

**IMPY.** The results agree with the predictions above, in that dCTP concentrations were elevated, dATP pools were decreased in cells treated with 0.5 mM and 1.0 mM IMPY, dGTP concentrations were increased at 8h in all IMPY-treated cells, and dTTP concentrations increased by 8h in cells treated with higher doses of IMPY (Fig. 3.16). However, the results disagree with the predictions in that these dGTP and dTTP pools were below control values at 16h and 24h. Also, unlike HU and 5HP, dCTP pools in CHO cells treated with 2.0 mM IMPY did not increase. Again, based on these predictions stated earlier, results from our experiments imply that the hypothesis is not correct for IMPY.

**INOX.** Deoxyribonucleoside triphosphates concentrations in INOX-treated cells were relatively unaffected (Fig. 3.17). Definitely, this is not consistent with our predictions and therefore, it can be tentatively concluded that INOX has no effect on ribonucleotide reductase in intact cells.

Another possible basis for our results would be that the four drugs considered are specific for the reduction of ADP only. If this were the case, then dCTP, dGTP and dTTP concentrations should increase as a result of a decrease in concentration of dATP. None of the results obtained with the four drugs was consistent with this prediction. Therefore, inhibition of the reduction of ADP in cells would not explain our observations.

However, it may be that our results reflect partial inhibition of ADP reduction together with complex regulation of the enzyme. If ADP reduction was partially inhibited, then dATP concentrations would decrease causing an increase in dCTP, dGTP and dTTP concentrations. These increases, in turn, would exert feedback effect on ribonucleotide reductase, *e.g.*, the increased dGTP would cause UDP reduction to increase and CDP reduction to decrease, increased dTTP would stimulate GDP reduction but inhibit CDP reduction, but increased dCTP concentrations would have no effect on ribonucleotide reductase activity. Therefore, the overall effects of complex regulation plus partial inhibition of ADP reduction would be decreased dCTP concentration and elevated dGTP and dTTP concentrations.

**HU.** With HU-treated cells, there was no decrease in dCTP nor increase in dTTP or dGTP levels. Therefore, this suggestion of partial inhibition of ADP reduction and complex regulation is not consistent with the hypothesis for HU. The only data consistent with this prediction are increased dGTP levels at 8h after treatment with 200 $\mu$M HU, and decreased dATP concentrations.

**5HP.** Deoxyribonucleoside triphosphate pool size changes in 5HP-treated cells were also not consistent with the changes just predicted. At the lower concentrations of 5HP, there was no decrease in dCTP or elevation of dGTP and dTTP pool sizes. As with HU, the only data consistent with the prediction were decreased dATP and increased dGTP concentrations at the 8h time point with the highest dose of 5HP. As in the case of HU, according to this scheme, our results are not consistent with the hypothesis for 5HP, *i.e.*, 5HP does not appear to inhibit ribonucleotide reductase in intact cells.

**IMPY.** As with HU and 5HP, the elevated dCTP concentrations in cells treated with 0.5 mM and 1.0 mM IMPY are not consistent with the prediction. However, unlike HU and 5HP, IMPY-treated cells had elevated dGTP concentrations at all three doses of IMPY used as well as increased dTTP levels at 8h after treatment with 1.0 mM and 2.0 mM IMPY. Also, the dCTP concentrations of cells treated with 2.0 mM IMPY were not increased. Of the three drugs considered, the deoxyribonucleotide changes in IMPY-treated cells were closest to those predicted; however, but in view of the differences that exists, we cannot state that the hypothesis is correct for IMPY.

**INOX.** As mentioned earlier, INOX has no effect on deoxyribonucleotides and therefore, it was concluded that the hypothesis is not correct for INOX, *i.e.*, INOX does not inhibit ribonucleotide reductase in intact cells.

Throughout the discussion, we have found that the changes in deoxyribonucleotide pool sizes of HU, 5HP and IMPY-treated cells do not conform to any of the proposed schemes based on the behaviour of ribonucleotide reductase suggested by Thelander and Reichard [1] or on intact cell studies by Hunting and Henderson [5,79]. These three drugs have been shown to inhibit the M2 subunit specifically in studies using partially purified subunits. Hence, it would be expected that binding of drugs to the enzyme should lead to decreases in all four deoxyribonucleotides (assuming that these drugs are specific for ribonucleotide reductase in intact cells); however this does not appear to be so.

In the discussion so far, we have neglected any possible change in the subunit structure and conformation of ribonucleotide reductase as a result of drug binding. According to a recently proposed model [26], ribonucleotide reductase exists in various combinations of four separate M1 subunits, each specific for a particular ribonucleoside diphosphate, associated with each other but sharing a common M2 subunit. Inhibition of a particular M1 subunit may affect adjoining subunits, for example, by changing enzyme conformation resulting in inhibition of activity or change in affinity of the adjoining M1 subunit for the M2 subunit. Therefore, it is possible that these drugs may have some effect on the assembly of the subunits in cells.

For example, the binding of drug to the M2 subunit might greatly decrease its affinity for the $M_1$.ADP subunit and possibly for the $M_1$.UDP subunit too. In the event of decreased $M_1$.ADP affinity, $M_1$.CDP might bind in place of $M_1$.ADP and $M_1$.GDP might bind in place of $M_1$.UDP. This could explain increased dCTP levels and dGTP levels and decreased dATP levels, but it does not explain the observed fluctuations in deoxyribonucleotide concentrations observed over the 24h time period.

Although this model might explain some of our results, it must be stressed that its credibility still remains to be established. Furthermore, the information used to construct this model are from studies using partially purified enzymes and permeabilised cells. Moreover, binding of drugs to the M2 subunit of the enzyme in intact cells has never been demonstrated.

To summarise, we conclude that INOX does not inhibit ribonucleotide reductase in intact cells as there was almost no effect on deoxyribonucleotide pools in cells treated with this drug. With HU, 5HP, and IMPY, there were changes in deoxyribonucleoside triphosphate concentrations, but these changes do not seem to fit any of the suggested responses to inhibition of ribonucleotide reductase. It could be that ribonucleotide reductase in CHO cells is inhibited but the results were more complicated than can be explained based on the present knowledge of the enzyme. Alternatively, these drugs could be exerting their effects elsewhere in the cell than at ribonucleotide reductase.

In work done with HU, using mouse embryo cells [45] and mouse L929 cells[29], dCTP and dTTP pools were shown to increase, and dATP and dGTP pools to decrease. High concentrations of HU were used in these experiments; 1 mM and 10 mM in experiments using embryo cells, and 2mM HU in experiments using L929 cells. Only dCTP and dATP pools in CHO cells behaved similarly in the presence of lower concentrations of HU. It should be mentioned that at such high concentrations of HU, DNA repair processes are affected; studies are being carried out elsewhere on the relationship between this effect and deoxyribonucleotide concentrations [48]. In HL-60 cells treated with 1.0 mM IMPY for 12h, dATP and dGTP were decreased by 60% and 40% respectively, but there was no change in the pyrimidine

deoxyribonucleotides. Except for the decreased dATP levels, doxyribonucleotides in IMPY-treated CHO cells responded differently, *i.e.*, dCTP increased, but dGTP and dTTP increased sometimes. We obtained a greater decrease in dATP concentrations in CHO cells; ca. 80% decrease 8h after treatment with 1.0 mM IMPY.

**3.5 Metabolism of Radioactive Precursors.**

Ribonucleotide reductase catalyses the reduction of ribonucleotides to deoxyribonucleotides in cells; therefore if cells are incubated with radioactive ribonucleosides, with time these will be incorporated into ribonucleotides, and thence not only into RNA but also into deoxyribonucleotides and DNA. If inhibitors of ribonucleotide reductase are present, then a decrease in radioactivity in deoxyribonucleotides and DNA would be expected. This prediction has been tested by monitoring the metabolism of radioactive ribonucleoside precursors in cells which had been treated with drugs for 4h (see chapter 2 for details); it was hoped that at 4h we would observe changes in the rates of reaction of ribonucleotide reduction that would precede the biochemical and biological changes previously studied at 8h. Radioactivity in the acid-insoluble fraction and in acid-soluble nucleotides were determined, and pool size determinations of deoxyribonucleoside triphosphates were also carried out. Controls to which saline was added were run concurrently. In addition, as a control for the incorporation of deoxyribonucleoside triphosphates into DNA, cells were incubated with radioactive thymidine; this precursor was used because it bypasses ribonucleotide reductase.

These experiments involved several assumptions. Firstly, it was assumed that the incorporation of precursors into nucleotides, DNA and RNA was linear for 30 min; this has been demonstrated previously by Hunting and Henderson [79,5] using CHO-K1 cells. The second assumption was that addition of radioactive precursors did not significantly perturb the metabolism of the cell, and it has been demonstrated that much higher concentrations were needed to cause measurable ribonucleotide pool size changes (Henderson, J.F. and Lowe, J.K., unpublished results). Thirdly, ribonucleotide pools were not determined at 4h, however as there

was no change in ribonucleotide pools 8h after drug treatment, it was assumed that this was also true at 4h.

**Nucleotide concentrations.** Table 3.2 shows the deoxy- and ribonucleotide pool sizes of control and drug-treated cells at 4h and 8h respectively (ribonucleotide concentrations are from Fig. 3.11, 3.13, 3.15, 3.17). No corrections for cell volume changes were made, as this parameter was not appreciable altered at these times. Ribonucleotide pools were relatively unchanged in the drug-treated cells but in the deoxyribonucleotide pools, dATP was decreased greatly in HU, 5HP and IMPY-treated cells but increased in INOX-treated cells; similarly dGTP was increased in HU, 5HP and IMPY treated cells but decreased in INOX-treated cells; dTTP was increased in cells treated with 5HP and IMPY, but dCTP was unchanged.

**[2,8-$^3$H]Adenosine metabolism.** The data in Table 3.3 show that the four drugs did not have any appreciable effect on the incorporation of radioactive adenosine into ATP and RNA of CHO cells; deoxycoformycin was present in these experiments to inhibit deamination of adenosine. There was only a small increase in total dpm in ATP and RNA of HU, 5HP and IMPY-treated cells, and a decrease in INOX-treated cells. The specific activity of the ATP in INOX-treated cells was lower than other drug-treated cells.

Total radioactivity in dATP plus DNA (Table 3.3) was decreased in all four drug-treated cell populations. The effects of the drugs on the incorporation of precursor into dATP were relatively small, but incorporation into DNA was greatly decreased; only 21%, 44%, 15% and 53% radioactivity of control was incorporated into DNA of HU, 5HP, IMPY and INOX-treated cells, respectively. The specific activity of dATP pools of drug-treated cells was increased except for INOX-treated cells, where a 50% decrease was observed.

**[8-$^3$H]Guanosine metabolism.** As with the studies using radioactive adenosine, there were decreases in total radioactivity in GTP pools and RNA (Table 3.4) of INOX-treated cells when radioactive guanosine was used (*ca.* 76% of control); there was also a small decrease in IMPY-treated cells (*ca.* 92% of control), but there was no major effect on the incorporation of radioactive precursor into GTP pools or RNA in the HU and 5HP-treated cells. Again, the

**Table 3.2A. Ribonucleotide concentrations[1,*].**

| Drugs | ATP | GTP | CTP | UTP |
|---|---|---|---|---|
| | (nmoles per $10^6$ cells) | | | |
| Control | 6.27 | 1.04 | 0.63 | 2.17 |
| HU, 200$\mu$M | 8.32 | 1.24 | 0.75 | 2.84 |
| 5HP, 10$\mu$M | 7.57 | 1.16 | 0.80 | 2.75 |
| IMPY, 2mM | 5.62 | 0.93 | 0.57 | 1.83 |
| INOX, 200$\mu$M | 8.35 | 1.45 | 0.87 | 2.37 |

[1]Data from Fig. 3.12, 3.14, 3.16 and 3.18.
[*]at 8h.

**Table 3.2B. Deoxyribonucleotide concentrations[1].**

| Drugs | dATP | dGTP | dCTP | dTTP |
|---|---|---|---|---|
| | (pmoles per $10^6$ cells) | | | |
| Control | 24.9 | 18.1 | 43.5 | 72.7 |
| HU, 200$\mu$M | 0.9 | 22.6 | 31.0 | 55.9 |
| 5HP, 10$\mu$M | 4.3 | 40.1 | 54.9 | 131.0 |
| IMPY, 2mM | 4.8 | 42.6 | 41.6 | 147.0 |
| INOX, 200$\mu$M | 39.6 | 14.5 | 41.2 | 96.5 |

[1]at 4h.

**Table 3.3. Incorporation of radioactive adenosine into nucleotides, RNA and DNA.**

| Drugs | ATP dpm | Specific Activity dpm/pmole | RNA dpm | sub-total dpm | dATP dpm | Specific Activity dpm/pmole | DNA dpm | sub-total dpm | Total dpm |
|---|---|---|---|---|---|---|---|---|---|
| Control | 30,100,000 | 4800 | 3,040,000 | 33,140,000 | 736,000 | 29,600 | 229,000 | 965,000 | 34,100,000 |
| HU, 200μM | 33,700,000 | 4050 | 3,050,000 | 36,800,000 | 803,000 | 892,000 | 47,200 | 850,000 | 37,700,000 |
| 5HP, 10μM | 32,300,000 | 4270 | 2,890,000 | 35,200,000 | 791,000 | 184,000 | 99,800 | 891,000 | 36,100,000 |
| IMPY, 2mM | 31,800,000 | 5650 | 2,480,000 | 34,300,000 | 616,000 | 129,000 | 34,600 | 651,000 | 35,000,000 |
| INOX, 200μM | 24,900,000 | 2980 | 2,290,000 | 27,200,000 | 636,000 | 16,100 | 122,000 | 758,000 | 28,000,000 |

**Table 3.4. Incorporation of radioactive guanosine into nucleotides, RNA and DNA.**

| Drugs | GTP dpm | Specific Activity dpm/pmole | RNA dpm | sub-total dpm | dGTP dpm | Specific Activity dpm/pmole | DNA dpm | sub-total dpm | Total dpm |
|---|---|---|---|---|---|---|---|---|---|
| Control | 3,310,000 | 3180 | 3,040,000 | 6,350,000 | 48,400 | 2670 | 122,000 | 171,000 | 6,520,000 |
| HU, 200μM | 3,920,000 | 3160 | 2,620,000 | 6,540,000 | 68,200 | 3020 | 26,000 | 94,200 | 6,630,000 |
| 5HP, 10μM | 3,290,000 | 2840 | 3,550,000 | 6,840,000 | 72,500 | 1810 | 41,300 | 114,000 | 6,950,000 |
| IMPY, 2mM | 3,090,000 | 3320 | 2,760,000 | 5,850,000 | 63,300 | 1480 | 13,100 | 76,400 | 5,930,000 |
| INOX, 200μM | 2,320,000 | 1600 | 2,500,000 | 4,820,000 | 51,700 | 3565 | 44,600 | 96,300 | 4,900,000 |

specific activity of GTP pools was relatively unchanged except in cells treated with INOX, where the specific activity was 50% of control values.

There was a general increase in the incorporation of radioactive guanosine into dGTP pools of drug-treated cells and a uniform decrease of that into DNA (Table 3.4). IMPY-treated cells had the lowest radioactivity (17% of control), while 5HP and INOX-treated cells had the least decrease, *ca.* 40%, and HU caused decreased incorporation of precursor into DNA by approximately 30%. The overall effect was a decrease of approximately 50% in total radioactivity in dGTP plus DNA of drug-treated cells.

**[5-$^3$H]Cytidine metabolism.** Incorporation of radioactive cytidine into CTP pools was not appreciably affected by the four drugs (Table 3.5); for RNA, incorporation was decreased *ca.* 50% in cells exposed to 5HP. The reduction of CTP to dCTP and subsequent incorporation of dCTP into DNA was also decreased in HU, 5HP and IMPY-treated cells, as observed by the decreased total radioactivity in dCTP plus DNA (51%, 77%, and 37% of control, respectively). Low incorporation into DNA was the main contributing factor; as in experiments using [2,8-$^3$H]adenosine and [8-$^3$H]guanosine, incorporation of [5-$^3$H]cytidine into DNA of IMPY-treated cells was the lowest of the four drugs. Radioactivity in the dCTP pools of HU, 5HP and INOX-treated cells was elevated and it is this great elevation in the INOX-treated cells which gave an overall increase in the total radioactivity of dCTP plus DNA (155% of control).

**[5-$^3$H]Uridine metabolism.** Incorporation of [6-$^3$H]uridine into UTP pools of CHO cells was lowered by the four drugs (Table 3.6); 5HP decreased its incorporation by *ca.* 45% whereas the other three drugs decreased incorporation by *ca.* 25% - 30%. There was decrease of approximately 50% in the radioactivity in UTP plus RNA of 5HP treated-cells.

Uridine labelled in the 5-position, loses its label during conversion of deoxyuridine nucleotides to deoxythymidine nucleotides, but not during conversion to cytidine and deoxycytidine nucleotides. Hence radioactivity in DNA in these experiments comes from incorporation of deoxycytidine triphosphate. The total incorporation of radioactive precursors into dCTP plus DNA in the drug-treated cells were also decreased by *ca.* 25% - 50%; this

**Table 3.5. Incorporation of radioactive cytidine into nucleotides, RNA and DNA.**

| Drugs | CTP dpm | Specific Activity dpm/pmole | RNA dpm | sub-total dpm | dCTP dpm | Specific Activity dpm/pmole | DNA dpm | sub-total dpm | Total dpm |
|---|---|---|---|---|---|---|---|---|---|
| Control | 2,760,000 | 4380 | 3,540,000 | 6,300,000 | 81,300 | 1870 | 155,000 | 236,000 | 6,540,000 |
| HU, 200μM | 3,060,000 | 4070 | 3,320,000 | 6,380,000 | 92,400 | 2980 | 27,500 | 120,000 | 6,500,000 |
| 5HP, 10μM | 2,310,000 | 2890 | 1,610,000 | 3,920,000 | 124,300 | 2260 | 57,400 | 182,000 | 4,100,000 |
| IMPY, 2mM | 3,080,000 | 5400 | 3,240,000 | 6,320,000 | 72,500 | 1740 | 16,200 | 88,700 | 6,410,000 |
| INOX, 200μM | 2,940,000 | 3380 | 2,880,000 | 5,820,000 | 247,000 | 5990 | 118,000 | 365,000 | 6,190,000 |

**Table 3.6. Incorporation of radioactive uridine into nucleotides, RNA and DNA.**

| Drugs | UTP dpm | Specific Activity dpm/pmole | RNA dpm | sub-total dpm | dCTP dpm | Specific Activity dpm/pmole | DNA dpm | sub-total dpm | Total dpm |
|---|---|---|---|---|---|---|---|---|---|
| Control | 998,000 | 460 | 5,120,000 | 6,120,000 | 198,000 | 4560 | 56,100 | 254,000 | 6,370,000 |
| HU, 200μM | 688,000 | 242 | 4,550,000 | 5,240,000 | 129,000 | 5870 | 39,200 | 221,000 | 5,410,000 |
| 5HP, 10μM | 558,000 | 203 | 2,300,000 | 2,860,000 | 129,000 | 2350 | 17,800 | 147,000 | 3,010,000 |
| IMPY, 2mM | 734,000 | 401 | 4,300,000 | 5,030,000 | 199,000 | 4780 | 14,400 | 213,000 | 5,240,000 |
| INOX, 200μM | 771,000 | 326 | 4,150,000 | 4,920,000 | 181,000 | 4400 | 28,000 | 209,000 | 5,130,000 |

decrease was due mostly to low incorporation into DNA and also sometimes into dCTP; as in experiments with other precursors, DNA had the lowest radioactivity (18% of control) in IMPY-treated cells. As in the studies using radioactive cytidine, cells treated with 5HP incorporated 50% less radioactive precusor than controls.

**[methyl-$^3$H]Deoxythymidine metabolism.** The incorporation of deoxythymidine into the acid-soluble fraction and DNA is shown in Table 3.7. Total radioactivity in acid-soluble nucleotides was increased two-fold by HU, 5HP and IMPY-treated cells but was unchanged in INOX treated cells. Incorporation into DNA of drug-treated cells was decreased, however, with HU and 5HP causing the largest decrease, *ca.* 70%. In the presence of IMPY, total incorporation of radioactive deoxythymidine was decreased by 50%. Only cells exposed to HU had an increase in specific activity of dTTP pools.

In general, none of the four drugs had an appreciable effect on the incorporation of radioactivity into ribonucleotide or RNA, except for radioactive cytidine and uridine in 5HP-treated cells (which will be discussed later).

**HU.** Table 3.8 summarises the effect of each drug on the incorporation of radioactive precursors into deoxyribonucleotides and DNA. Radioactivity in all deoxyribonucleotides of HU-treated cells was increased except for dTTP (from [5-$^3$H] uridine), but decreased in DNA. When both deoxyribonucleotides and DNA was considered, it is apparent that HU produced an overall decrease in radioactivity in all cases.

**5HP.** As with the incorporation of radioactivity into deoxyribonucleotides of HU-treated cells, radioactivity in all deoxyribonucleotides in 5HP-treated cells was increased except for dTTP (from [5-$^3$H]uridine); dTTP derived from [methyl-$^3$H]deoxythymidine had the greatest increase in radioactivity compared to the other deoxyribonucleotides. 5HP decreased incorporation of all precursors into DNA, and the overall effect was a decrease in radioactivity in deoxyribonucleotides plus DNA.

**IMPY.** Incorporation of radioactive adenosine and cytidine into deoxyribonucleotides was decreased, but with radioactive guanosine and deoxythymidine incorporation was increased,

**Table 3.7. Incorporation of radioactive deoxythymidine into nucleotides, RNA and DNA.**

| Drugs | dTDP | dTTP | Specific Activity | sub-total | DNA | Total |
|---|---|---|---|---|---|---|
| | dpm | dpm | dpm/pmole | dpm | dpm | dpm |
| Control | 50,400 | 1,550,000 | 21,300 | 1,600,000 | 4,870,000 | 6,470,000 |
| HU, 200$\mu$M | 117,000 | 4,220,000 | 75,400 | 4,340,000 | 1,530,000 | 5,870,000 |
| 5HP, 10$\mu$M | 63,100 | 2,990,000 | 22,900 | 3,050,000 | 1,770,000 | 4,820,000 |
| IMPY, 2mM | 76,200 | 2,920,000 | 19,900 | 3,000,000 | 393,000 | 3,390,000 |
| INOX, 200$\mu$M | 42,300 | 1,570,000 | 16,300 | 1,610,000 | 3,040,000 | 4,650,000 |

**Table 3.8. Relative effects of drugs on the incorporation of radioactive precursors into deoxyribonucleotides and DNA[1].**

| Drug | Precursor | dNTP | DNA | dNTP + DNA |
|---|---|---|---|---|
| | | (% of Control) | | |
| HU | AR | 109 | 21 | 88 |
| | GR | 141 | 21 | 55 |
| | CR | 114 | 18 | 51 |
| | UR | 92 | 70 | 87 |
| | TdR | 272 | 31 | 90 |
| 5HP | AR | 107 | 44 | 92 |
| | GR | 150 | 34 | 67 |
| | CR | 153 | 37 | 77 |
| | UR | 65 | 32 | 58 |
| | TdR | 193 | 36 | 74 |
| IMPY | AR | 84 | 15 | 67 |
| | GR | 131 | 11 | 44 |
| | CR | 89 | 10 | 37 |
| | UR | 100 | 25 | 84 |
| | TdR | 190 | 81 | 52 |
| INOX | AR | 86 | 53 | 79 |
| | GR | 107 | 37 | 56 |
| | CR | 304 | 76 | 155 |
| | UR | 91 | 50 | 82 |
| | TdR | 102 | 62 | 72 |

[1]Data recalculated from Fig. 3.3 - 3.7.

and with uridine there was no change. Radioactivity in DNA of IMPY-treated cells was decreased with all precursors. As with HU and 5HP, IMPY-treated cells had decreased radioactivity when deoxyribonucleotides and DNA were added together.

**INOX**. Incorporation of radioactive precursors into dATP and dTTP (from [5-$^3$H]uridine) was decreased and in contrast to the other three drugs, the greatest increase was in dCTP and not in dTTP. Also, radioactivity in DNA of INOX-treated cells were decreased and in general, the total radioactivity of deoxyribonucleotides and DNA was decreased except when radioactive cytidine was used.

If it is assumed that radioactivity in deoxyribonucleoside triphosphate pools plus DNA equals total ribonucleotide reduction in cells, then these four drugs do inhibit ribonucleotide reductase in intact cells, though to varying degrees (Table 3.8). In order for this equation to be true, two assumptions have to be made: that entry of radioactive precursors into cells was unaffected by the four drugs, and that there was no breakdown of radioactive deoxyribonucleotides and DNA.

The extent of entry of precursors in the presence of drugs can be determined by adding up all counts in both acid-soluble and acid insoluble fractions. The average total incorporation for HU, IMPY and INOX was *ca.* 5 - 15% below control values (data not shown). However, some variation in total incorporation as a result of the many steps involved in the preparation of these extracts as well as some biological variation should be expected; hence, it can be tentatively concluded that HU, IMPY and INOX did not appreciably affect the entry of precursors into cells.

In the case of 5HP, larger fluctuations in total radioactivity were observed, *e.g.*, total radioactive adenosine and guanosine in cells was *ca.* 100% of control whereas total radioactive deoxythymidine was *ca.* 70% and those of radioactive cytidine and uridine were *ca.* 50% of control. It was also noted that exposure of cells to a final ethanol concentration of 0.75% (the solvent for this drug) gave inconsistent incorporation of radioactive precursors, therefore these effects observed with 5HP might be caused partially by ethanol; this concentration of ethanol

was *ca.* 10-fold higher than that used in growth inhibition experiments.

Any major amount of breakdown of deoxyribonucleotides or DNA to deoxyribonucleosides in drug-treated cells can be detected by comparing the radioactivity in the total acid-soluble extract to that in the nucleotide triphosphates. Under normal circumstances, *ca.* 90% of radioactivity in the acid-soluble extract is in triphosphates (except for uridine, where radioactivity was also incorporated into UDP sugars), with the remaining 10% in deoxyribonucleosides and di- and monophosphates [79], only. In these experiments, this was true for all samples, both control and drug-treated (data not shown). This implies that there was no appreciable breakdown of deoxyribonucleotides and DNA.

If ribonucleotide reductase in intact cells is inhibited by these drugs, as implied by the conclusion stated earlier, then one would expect to find a decreased level of radioactivity in deoxyribonucleoside triphosphate pools with a subsequent decrease in radioactivity in DNA. Contrary to this expectation, radioactivity in deoxyribonucleoside triphosphates was elevated in many cases, *e.g.*, dGTP of HU, 5HP and IMPY-treated cells, dCTP of HU, 5HP and INOX-treated cells, while decreased in some *e.g.*, dATP of IMPY and INOX-treated cells, dCTP of IMPY-treated cells and dCTP (synthesised from [5-$^3$H]UR) in HU, 5HP and INOX-treated cells (Table 3.8). A possible reason for the increased deoxyribonucleotide pools might be alternative pathways of deoxyribonucleotide synthesis from the radioactive precursors, although this is highly unlikely.

In contrast to the increased level of radioactivity in deoxyribonucleoside triphosphates, radioactivity in DNA of drug-treated cells was decreased. The possibility of DNA breaking down to deoxyribonucleosides has been ruled out, and moreover, it has been shown in radioactive precursor studies, that newly synthesised DNA is relatively stable [79].

Decreased radioactivity in DNA of drug-treated cells could potentially be due to inhibition of DNA polymerase by the drugs. To investigate this possibility, the deoxyribonucleoside triphosphate assay (described in chapter 2) was performed in the presence of the four drugs, at concentrations used in the intact cell studies; not one of the drugs

inhibited DNA polymerase under these conditions (data not shown). Although there was no inhibition of DNA polymerase under these assay conditions, the experiments with radioactive deoxythymidine raises this possibility (Table 3.8); there do not seem to be any explanations for this discrepancy.

Therefore, having discounted the breakdown of DNA as a possible cause of low levels of radioactivity in DNA, and if there is no inhibition of DNA polymerase, we propose that the lowered radioactivity in DNA of drug-treated cells is a direct result of inhibition of ribonucleotide reductase. Our results, indicate that HU gave a consistent 70% - 80% inhibition of ribonucleotide reductase from studies using radioactive adenosine, guanosine, and cytidine, but only 30% inhibition with uridine. 5HP inhibited incorporation of all four precursors by approximately 60% - 70%. IMPY gave results similar to those with 5HP. Of the four drugs studied, INOX had the least effect on incorporation of precursors into DNA, *ca.* 50% inhibition was obtained.

If the lowered radioactivity in DNA of drug-treated cells is a direct result of inhibition of ribonucleotide reductase and the radioactivity in deoxyribonucleotides is high, this could imply that not all of the acid-soluble deoxyribonucleotide pools are available to the cell for synthesis of DNA; this suggests that deoxyribonucleotide pools are compartmentalised. In their review on compartmentation, Moyer and Henderson [86] reported that the rate of DNA synthesis may be affected by compartmentation of deoxyribonucleotide pools.

In our attempt to look for more evidence of compartmentation, we calculated the apparent rates of DNA synthesis using the method of Hunting and Henderson [79,87], in which it was assumed that if there was no compartmentation or metabolic channeling, the rates of synthesis would be similar regardless of the precursor used. In contrast to Hunting and Henderson's results with a different line of CHO cells, our data (table 3.9) gave quite different rates of DNA synthesis for each precursor in control cells, therefore implying that compartmentation may be a factor in our experiments with CHO-K1-C3 cells.

**Table 3.9. Effects of drugs on rates of radioactive dNTP incorporation into DNA.**

| Precursor | Treatment | Rate of DNA Synthesis (pmoles/min per $10^6$ cells) |
|---|---|---|
| [2,8-$^3$H]AR | None | 0.258 |
| | HU | 0.002 |
| | 5HP | 0.018 |
| | IMPY | 0.009 |
| | INOX | 0.254 |
| [8-$^3$H]GR | None | 1.530 |
| | HU | 0.287 |
| | 5HP | 0.761 |
| | IMPY | 0.294 |
| | INOX | 0.417 |
| [5-$^3$H]CR | None | 2.760 |
| | HU | 0.308 |
| | 5HP | 0.845 |
| | IMPY | 0.310 |
| | INOX | 0.656 |
| [5-$^3$H]UR | None | 0.410 |
| | HU | 0.223 |
| | 5HP | 0.252 |
| | IMPY | 0.100 |
| | INOX | 0.212 |
| [methyl-$^3$H]TdR | None | 7.620 |
| | HU | 0.677 |
| | 5HP | 2.578 |
| | IMPY | 0.657 |
| | INOX | 6.212 |

HU, 200 μM; 5HP, 10 μM; IMPY, 2 mM; INOX, 200 μM.

If there is compartmentation, then the decreased apparent rates of DNA synthesis for drug-treated cells may be a consequence of limited substrate availability, with this substrate pool being distinct from the total pool. Therefore, due to this distinction in substrate pools, the effect of drugs on DNA synthesis appears to be disproportionate to the effect on deoxyribonucleotide triphosphate pools. This inconsistency between the response of DNA synthesis and pool sizes to drugs has also been observed by other researchers [86].

If nucleotide pools are compartmentalised in CHO-K1-C3 cells and the lowered radioactivity in DNA is a direct result of inhibition of ribonucleotide reductase, then this would imply that our hypothesis is correct for the four drugs. However, until more evidence has been gathered to show that compartmentation of deoxyribonucleotide pools in this particular cell line does occur, we cannot conclusively state that these drugs inhibit ribonucleotide reductase in the intact cells studied. If there is no compartmentation, and considering the fact that deoxyribonucleotide concentrations were sometimes increased, then the specificity of these drugs would have to be seriously questioned.

## 4. CONCLUSIONS.

The aim of this project was to test two hypotheses: (1) that all four drugs do inhibit ribonucleotide reductase in intact cells; and (2) that growth inhibition is a direct and sole consequence of inhibition of ribonucleotide reductase.

Table 4.1 summarises the predicted and the observed results for each of the drugs and each of the experimental parameters studied. As INOX had no effect on deoxyribonucleotide concentrations in intact cells under our experimental conditions, we concluded that it does not inhibit ribonucleotide reductase inside cells and therefore, both hypotheses are not correct for INOX. For HU, 5HP and IMPY, the results did not completely agree with predictions and therefore, it was also suggested that the hypothesis is not correct for these three drugs. The effects of HU and 5HP were similar in these experiments, but the effects of IMPY was somewhat different: cells were synchronised in late $G_1$-early S (the predicted result), the dTTP pool was sometimes increased at the highest dose of drug, incorporation of radioactivity into dTTP (from [5-$^3$H]uridine) was not affected, but incorporation into dCTP was decreased. In general, IMPY was most consistent with the predicted results. Although the results with HU, 5HP and IMPY are not completely consistent with the hypothesis, the fact that deoxyribonucleotide pools were perturbed suggests that they did inhibit ribonucleotide reductase but that the expression of this inhibition was not as straightforward as expected. The observed growth inhibition with HU, 5HP and IMPY is related to inhibition of DNA synthesis, however, there is no evidence that inhibition of DNA synthesis is entirely due to inhibition of ribonucleotide reductase.

**Table 4.1. Summary of Predicted and Observed Results.**

| Drug | Cell Volume | Cell Cycle | dNTP Pools | Precursor →dNTP | Precursor →DNA |
|---|---|---|---|---|---|
| Predicted[1] results | increase | late $G_1$-early S | decrease in all 4 dNTP | decrease in all 4 dNTP | decrease |
| HU | increase | S | ↓ dATP<br>↑ dCTP, dGTP[2]<br>- dTTP | ↓ dTTP ([$^3$H]UR)<br>↑ dGTP, dCTP<br>↑ dTTP<br>- dATP | decrease |
| 5HP | increase | S | ↓ dATP<br>↑ dCTP, dGTP[2]<br>- dTTP | ↓ dTTP ([$^3$H]UR)<br>↑ dGTP, dCTP<br>↑ dTTP<br>- dATP | decrease |
| IMPY | increase | late $G_1$-early S | ↓ dATP<br>↑ dCTP, dGTP<br>↑ dTTP[2] | ↓ dATP, dCTP<br>↑ dGTP, dTTP<br>- dTTP ([$^3$H]UR) | decrease |
| INOX | increase | $G_2$M | no change in all 4 dNTP | ↓ dATP<br>↓ dTTP ([$^3$H]UR)<br>↑ dCTP<br>- dGTP, dTTP | decrease |

[1] simplest case.
[2] dNTP effect observed with highest drug concentration only.
[3] -; indicate no change.

**References.**


1. Thelander, L., & Reichard, J. (1979) Reduction of Ribonucleotides. *Ann. Rev. Biochem.* 48, 133-158.
2. Holmgren, A. (1981) Regulation of Ribonucleotide Reductase. *Curr. Top. Cell. Reg.* 19, 47-76.
3. Hunting, D., & Henderson, J.F. (1983) Models of the Regulation of Ribonucleotide Reductase and their Evaluation in Intact Mammalian Cells. *CRC Crit. Rev. Biochem.* 13, 325-348.
4. Hunting, D., Hordern. J., & Henderson, J.F. (1981) Effects of Altered Ribonucleotide Concentrations on Ribonucleotide Reduction in Intact Chinese Hamster Ovary Cells. *Can. J. Biochem.* 59, 821-829.
5. Hunting, D., Hordern, J., & Henderson, J.F. (1981) Quantitative Analysis of Purine and Pyrimidine Metabolism in Chinese Hamster Ovary Cells. *Can. J. Biochem.* 59, 838-847.
6. Hards, R.G., & Wright, J.A. (1983) Assay of Ribonucleotide Reductase Activity in Intact Permeabilized Hamster Cells : An Evaluation. *Arch. Biochem. Biophys.* 220, 576-583.
7. Kucera, R., & Paulus, H. (1982) Studies on Ribonucleoside-diphosphate Reductase in Permeable Animal Cells. *Arch. Biochem. Biophys.* 214, 114-123.
8. Hunting, D., & Henderson, J.F. (1981) Regulation of Ribonucleotide Reduction by Deoxyribonucleotides in Intact Chinese Hamster Ovary Cells. *Can. J. Biochem.* 59, 830-837.
9. Kucera, R., Brown, C.L., & Paulus, H. (1983) Cell Cycle Regulation of Ribonucleoside Diphosphate Reductase Activity in Permeable Mouse L Cells and in Extracts. *J. Cell. Physiol.* 117, 158-168.
10. Larsson, A., & Reichard, P. (1966) Enzymatic Synthesis of Deoxyribonucleotides. *J. Biol. Chem.* 241, 2533-2539.

11. Beck, W.S. (1967) Regulation of Cobamide-dependent Ribonucleotide Reductase by Allosteric Effectors and Divalent Cations. *J. Biol. Chem.* 242, 3148-3158.

12. Cory, J.G., & Mansell, M.M. (1975) Comparison of the Cytidine 5'-Diphosphate and Adenosine 5'-Diphosphate Reductase Activities of Mammalian Ribonucleotide Reductase. *Cancer Res.* 35, 2327-2331.

13. Cory, J.G., Mansell, M.M., & Whitford, T.W. (1976) Control of Ribonucleotide Reductase in Mammalian Cells. *Adv. Enz. Regul.* 14, 45-62.

14. Peterson, D.M., & Moore, E.C. (1976) Independent Fluctuations of Cytidine and Adenosine Diphosphate Reductase Activities in Cultured Chinese Hamster Fibroblast. *Biochim. Biophys. Acta.* 432, 80-91.

15. Walters, R.A., Tobey, R.A., & Ratliff, R.L. (1973) Cell-Cycle Dependent Variations of Deoxynucleoside Triphosphate Pools in Chinese Hamster Cells. *Biochim. Biophys. Acta.* 319, 336-347.

16. Chang, C.H., & Cheng, Y.C. (1979) Demonstration of Two Components and Association of Adenosine Diphosphate—Cytidine Diphosphate Reductase from Cultured Human Lymphoblast Cells (Molt-4F). *Cancer Res.* 39, 436-442.

17. Eriksson, S., Thelander, L., & Akerman, M. (1979) Allosteric Regulation of Calf Thymus Ribonucleoside Diphosphate Reductase. *Biochemistry* 18, 2948-2952.

18. Chang, C.H., & Cheng, Y.C. (1979) Substrate Specificity of Human Ribonucleotide Reductase from Molt-4F Cells. *Cancer Res.* 39, 5081-5086.

19. Cory, J.G. (1978) Properties of Ribonucleotide Reductase from Ehrlich Tumor Cells ; Multiple Nucleoside Diphosphate Activities and Reconstitution of Activity from Components. *Adv. Enz. Regul.* 17, 115 .

20. Cory, J.G., & Fleischer, A.E. (1982) The Molecular Weight of Ehrlich Tumor Cell Ribonucleotide Reductase and its Subunits : Effector-Induced Changes. *Arch. Biochem. Biophys.* 217, 546-551.

21. Youndale, T., & MacManus, J.P. (1979) Separation of a Subunit Necessary for CDP Reductase from other Ribonucleotide Reductase Activities of Regenerating Rat Liver. *Biochem. Biophys. Res. Commun.* 89, 403-409.

22. Youndale, T., MacManus, J.P., & Whitfield, J.F. (1982) Rat Liver Ribonucleotide Reductase: Separation, Purification and Properties of Two Nonidentical Subunits. *Can. J. Biochem.* 60, 463-470.

23. Morita, T., Murayama, J., Shimizu, Y., Moriyama, Y., & Fujimura, S. (1984) Preparation of Cytidine Diphosphate Reductase from Rat Yoshida Ascites Sarcoma. *Biochem. Biophys. Res. Commun.* 118, 212-216.

24. Larsson, A. (1969) Ribonucleotide Reductase from Regenerating Rat. *Eur. J. Biochem.* 11, 113-121.

25. Hards, R.G., & Wright, J.A. (1984) Ribonucleotide Reductase Activity in Intact Mammalian Cells: Stimulation of Enzyme Qctivity by $MgCl_2$, Dithiothreitol, and Several Nucleotides. *Arch. Biochem. Biophys.* 231, 9-16.

26. Hards, R.G., & Wright, J.A. (1984) Regulation of Ribonucleotide Reductase Activity in Intact Mammalian Cells. *Arch. Biochem. Biophys.* 231, 17-28.

27. Elford, H.L., Freese, M., Passamani, E., & Morris, H.P. (1970) Ribonucleotide Reductase and Cell Proliferation. *J. Biol. Chem.* 245, 5228-5233.

28. Cheng, Y.C., Chang, C.H., Williams, M.V., Cass, C.E., & Patterson, A.R.P. (1977) Fluctuations of Ribonucleotide Reductase Activity and Deoxyribonucleotide Pools in Synchronized Hela cells. *Proc. Am. Ass. Cancer. Res.* 18, 185.

29. Adams, R.L.P., Berryman, S., & Thomson, A. (1971) Deoxynucleoside Triphosphate Pools in Synchronized and Drug-inhibited L929 Cells. *Biochim. Biophys. Acta.* 240, 455-462.

30. Hordern, J., & Henderson, J.F. (1982) Comparison of Purine and Pyrimidine Metabolism in G1 and S Phases of HeLa and Chinese Hamster Ovary Cells. *Can. J. Biochem.* 60,

422-433.

31. Cory, J.G. (1983) Role of Ribonucleotide Reductase in Cell Division. *Pharmac. Ther.* 21, 265-276.

32. Steams, B., Losee, K.A., & Bernstein, J. (1963) Hydroxyurea: A New Type of Potential Antitumor Agents. *J. Med. Chem.(abstr)* 6, 201.

33. Krakoff, I.H. (1975) Clinical and Pharmacologic Effects of Hydroxyurea. In *Handbook of Experimental Parmacology*. A.C. Sartorelli & D.G. Johns (eds.), Vol.38, pt II, pp 793-807. Springer Verlag, Berlin.

34. Schwartz, H.S., Garofalo, M., Sternberg S.S., & Philips, F.S. (1967) Hydroxyurea: Inhibition of Deoxyribonucleic Acid Synthesis in Regenerating Liver of Rats. *Cancer Res.* 25, 1867-1870.

35. Young, C.W., & Hodas, S. (1964) Hydroxyurea: Inhibitory Effect on DNA Metabolism. *Science* 146, 1172-1174.

36. Mohler, W.C. (1964) Cytoxicity of Hydroxyurea (NSC-32065) Reversible by Pyrimidine Deoxyribosides in a Mammalian Cell Line Grown In Vitro. *Cancer Chemother. Rep.* 34, 1-6.

37. Gale, G.R., Kendall, S.M., McClain, H.H., & Dubois, S. (1964) Effect of Hydroxyurea on *Pseudomonas aeroginosa*. Cancer Res. 24, 1012-1019.

38. Rosenkranz, H.S., Garro, A.J., Levy, J.A., & Carr, H.S. (1966) Studies with Hydroxyurea. I. The Reversible Inhibition of Bacterial DNA Synthesis and the Effect of Hydroxyurea on the Bacterial Action of Streptomycin. *Biochim. Biophys. Acta.* 114, 501-515.

39. Murphy, M.L., & Chaube, S. (1964) Preliminary Survey of Hydroxyurea (NSC-32065) as a Teratogen. *Cancer Chemother. Rep.* 40, 1-7.

40. Adams, R.L.P., & Lindsay, J.G. (1967) Hydroxyurea. Reversal of Inhibition and Use as a Cell-Synchronizing Agent. *J. Biol. Chem.* 25, 1314-1317.

41. Skipper, H.E. (1962) Leucocyte Kinetics—Leukemia and Lymphoma. in *Leukemia and Lymphoma*. Chicago. Year Book Medical Publishers.

42. Krakoff, I.H., Brown, N.C., & Reichard, P. (1968) Inhibition of Ribonucleoside Diphosphate Reductase by Hydroxyurea. *Cancer Res.* 28, 1559-1565.

43. Brown, N.C., Eliasson, R., Reichard, P., & Thelander, L. (1968) Nonheme Iron as a Cofactor in Ribonucleotide Reductase From *E. coli*. *Biochem. Biophys. Res. Commun.* 30, 522-527.

44. Moore, E.C. (1969) The Effects of Ferrous Ion and Dithioerythritol on Inhibition by Hydroxyurea of Ribonucleotide Reductase. *Cancer Res.* 29, 291-295.

45. Skoog, L., & Nordenskjold, B. (1971) Effects of Hydroxyurea and 1-$\beta$-D-Arabinofuranosyl-cytosine on Deoxyribonucleotide Pools in Mouse Embryo Cells. *Eur. J. Biochem.* 19, 81-89.

46. Plageman, P.G.W., & Erbe, J. (1974) Cellular Conversions of Deoxyribonucleosides by Novikoff Rat Hepatoma Cells and Effects of Hydroxyurea. *J. Cell. Physiol.* 83, 321-336.

47. Young, C.W., Schochetman, G., & Karnofsky, D.A. (1967) Hydroxyurea-induced Inhibition of Deoxyribonucleotide Synthesis: Studies in Intact Cells. *Cancer Res.* 27, 526-531.

48. Clarkson, J. (1978) Enhancement of Repair Replication in Mammalian Cells by Hydroxyurea. *Mutation Res.* 52, 273-284.

49. Karon, M., & Benedict, W.F. (1972) Chromatid Breakage: Differential Effect of Inhibitors of DNA Synthesis during G2 Phase. *Science* 178, 62.

50. Hansson, K., & Hartley-Asp, B. (1981) Potentiation by Hydroxyurea in G2-Prophase of Thiotepa- and Bleomycin-induced Chromosomal Aberrations in Chinese Hamster Cells. *Hereditas*, 94, 21-27.

51. Agrawal, K.C., & Sartorelli, A.C. (1975) $\alpha$-(*N*)-Heterocyclic Carboxaldehyde Thiosemicarbazones. In *Handbook of Experimental Pharmacology*, A.C. Sartorelli and

D.G. Johns (eds.), vol. 38, pt II, pp 793-807. Springer Verlag, Berlin.

52. Brockman, R.W., Thomson, J.R., Bell, M.J., & Skipper, H.E. (1956) Observations on the Antileukemic Activity of Pyridine-2-carboxaldehyde Thiosemicarbazone and Thiocarbohydrazone. *Cancer Res.* 16, 167-170.

53. Agrawal, K.H., & Sartorelli, A.C. (1978) The Chemistry and Biological Activity of a-(N)-Heterocyclic Carboxaldehyde Thiosemicarbazones. *Prog. Med. Chem.* 15, 321-366.

54. Krakoff, I.H., Etcubanas, E., Tan, C., Mayer, K., Bethune, V., & Burchenal, J.H. (1974) *Cancer Chemother. Rep.* 58, 207-212.

55. Sartorelli, A.C. (1967) Effects of Chelating Agents upon the Synthesis of Nucleic Acids and Proteins: Inhibition of DNA Synthesis by I-Formylisoquinoline Thiosemicarbazone. *Biochem. Biophys. Res. Commun.* 27, 26-32.

56. Booth, B.A., Moore, E.C., & Sartorelli, A.C. (1971) Metabolic Effects of Some Tumor-inhibitory Pyridine Carboxaldehyde Thiosamicarbazones. *Cancer Res.* 31, 228-234.

57. Bhuyan, B.K., Schmidt, L.G., & Fraser, T.J. (1972) Cell Cycle Phase Specificity of Antitumor Agents. *Cancer Res.* 32, 398-407.

58. Booth, B.A., Moore, E.C., & Sartorelli, A.C. (1971) Inhibition of Deoxyribonucleotide Synthesis by Pyridine Carboxaldehyde Thiosemicarbazones. *Cancer Res.* 31, 235-238.

59. Brockman, R.W., & Shaddix, S. (1970) Inhibition of Ribonucleotide Reductase, DNA Synthesis and Leukemia by Guanozole. *Cancer Res.* 30, 2358-2368.

60. Sartorelli, A.C. (1969) Some Approaches to the Therapeutic Exploitation of Metabolic Sites of Vulnerability of Neoplastic Cells. *Cancer Res.* 29, 2292-2299.

61. Moore, E.C. & Sartorelli, A.C. (1984) Inhibition of Ribonucleotide Reductase by α-(N)-Heterocyclic Carboxaldehyde Thiosemicarbazones. *Pharmac. Ther.* 24, 439-447.

62. Saryan, L.A., Ankel, E., Krishnamurti, C. & Petering, D.H. (1979) Comparative Cytotoxic and Biochemical Effects of Ligands and Metal Complexes of α-(N)-Heterocyclic Carboxaldehyde Thiosemicarbazones. *J. Med. Chem.* 22, 1218-1221.

63. Thelander, L., & Gralund, A. (1983) Mechanism of Inhibiton of Mammalian Ribonucleotide Reductase by the Iron Chelate of 1-Formylisoquinoline Thiosemicarbazone. *J. Biol. Chem.* 258, 4063-4066.

64. Brockman, R.W., Shaddix, S.C., Carpenter, J.W., & Dubois, N.F., (1978) Struck, R.F. Studies on the Mechanism of Action of 2,3-dihydro-1H-imidazolo[1,2-*b*]imidazole (NSC 51143) and Evidence of its Metabolism in vivo,. *Proc. Am. Assoc. Cancer Res.* 19, 21.

65. Cory. J.G., & Fleischer, A.E. (1980) Mode of Inhibition of Tumor Cell Ribonucleotide Reductase by 2,3-dihydro-1*H*-pyrazolo[1,2-*A*]imidazole (NSC 51143) *Cancer Res.* 40, 3891-3894.

66. Ennis, H.L., Moller, L., Wang, J.L., & Selawry, O.S. (1971) 2,3-Dihydro-1*H*-imidazo[1,2-*b*]pyrazole : A New Inhibitor of Deoxyribonucleic Acid Synthesis. *Biochem. Pharmacol.* 20, 2639-2646.

67. Grant, S., Bhalla, K., Rauscher, F., & Cadman, E. (1983) Potentiation of 1-$\beta$-D-Arabinofuranosylcytosine Metabolism and Cytotoxicity by 2,3-Dihydro-1*H*-imidazolo[1,2-*b*]pyrazole in the Human Promylelocytic Leukemic Cell, HL-60. *Cancer Res.* 43, 5093-5700.

68. Beer, C.T., Kajiwara, K., Mueller, G.C. (1974) Synchronization of Hela cells with 2,3-dihydro-1H-imidazo[1,2-*b*] pyrazole. *Biochem. Pharmacol.* 23, 1115-1122.

69. Krishan, A., Paika, K.D., & Frei, E. (1976) Cell Cycle Synchronization of Human Lymphoblast Cells in vitro by 2,3-dihydro-1*H*-imidazo[1,2-*b*]pyrazole. *Cancer Res.* 36, 138-142.

70. Ganapathi, R., & Krishan, A. (1980) Effect of 2,3-dihydro-1*H*-imidazo[2,3-*b*]pyrazole on the Proliferation of Mouse Leukemic and Normal Cells in vivo. *Cancer Res.* 40, 1103-1108.

71. Allen, L.M., & Thornwaite, J.T. (1980) Studies on Pharmacology and Cytokinetics of 2,3-dihydro-1*H*-imidazo[2,3-*b*] pyrazole (NSC 51143) with P815 Mastocytoma Cells.

*Cancer Res.* 40, 4059-4065.

72. Cory, J.G., Mansell, M.M., & Whitford, T.W. (1976) Inhibition of Ribonucleotide Reductase Activity and Nucleic Acid Synthesis in Tumor Cells by the Dialdehyde Derivative of Inosine (NSC 118994) and Inosinic Acid. *Cancer Res.* 36, 3166-3170.
73. Chiuten, D.F., Vosika, G.J., & Shaw, M.T., Boiron, M., Gisselbrecht, C., Marty, M., Higgins, G., & Muggia, F.M. (1981) Clinical Trials with Diglycoaldehyde (NSC 118994) : Review and Reasons for Withdrawal from Clinical Trial. *Anticancer Res.* 1, 121-124.
74. Sato, A, & Cory, J.G. (1981) Evaluation of Combinations of Drugs that Inhibit Ehrlich Tumor Cell Ribonucleotide Reductase. *Cancer Res.* 41, 1637-1641.
75. Cory, J.G., & Fleischer, A.E. (1979) Specific Inhibitors Directed at the Individual Components of Ribonucleotide Reductase as an Approach to Combination Chemotherapy. *Cancer Res.* 39, 4600-4604.
76. Plageman, P.G.W., Graff, J.C., & Behrens, M. (1977) Mechanism of Action of Inosine Dialdehyde (NSC 118994) in the Inhibition of Proliferation of Tumor Cells in Culture. *Cancer Res.* 37, 2188-2195.
77. Cory, J.G., Parker, S.H., & Fox, C.S. (1978) Inhibition of RNA Synthesis in Ehrlich Tumor Cells by the Dialdehyde Derivative of Inosine (NSC 118994). *Cancer Res.* 38, 815-822.
78. Bhuyan, B.K., & Fraser, T.J. (1974) Cytoxicity of Antumor Agents in a Synchronous Mammalian Cell System. *Cancer Chemother. Rep.* 58, 149-155.
79. Hunting, D. (1981) PhD. Dissertation. "*Regulation of Deoxyribonucleotides Metabolism.*" University of Alberta, Edmonton, Alta.
80. Khym, J.X. (1975) Neutralization of Perchloric or Trichloroacetic acid Extracts Using Alamine. *Clin. Chem.* 21, 1245-1252.
81. Hunting, D., & Henderson, J.F. (1981) Determination of Deoxyribonucleoside Triphosphates Using DNA Polymerase: a Critical Evaluation. *Can. J. Biochem.* 59, 723-727.

82. Crabtree, G.W. & Henderson, J.F. (1971) Rate-limiting Steps in the Interconversion of Purine Ribonucleotides in Ehrlich Ascites Tumor Cells *in Vitro*. *Cancer Res.* 31, 985-991.

83. Schwartz, P.M., & Drach, J.C. (1975) Separation of Arabinosyl, Ribosyl, and Deoxyribosyl Purine Nucleotides by Thin-layer Chromatography. *J. Chromat.* 106, 200-203.

84. Seipel, S., & Reichert, U. (1977) Two-dimensional Thin-layer Chromatography-autoradiography of Intracellular Purine Interconversion Products. *J. Chromat.* 135, 485-588.

85. Sato, A., Carter, G.L., Bacon, P.E., & Cory, J.G. (1982) Effects of Combinations of Drugs Having Different Modes of Action at the Ribonucleotide Reductase Site on Growth of L1210 Cells in Culture. *Cancer Res.* 42, 4353-4357.

86. Moyer, J.D., & Henderson, J.F. (1984) Compartmentation of Intracellular Nucleotides in Mammalian Cells. *CRC Crit. Rev. Biochem.*, in press.

87. Hunting, D., & Henderson, J.F. (1982) Relationship Between Ribo- and Deoxyribonucleotide Concentrations and Biological Parameters in Cultured Chinese Hamster Ovary Cells. *Biochem. Pharmacol.* 31, 1109-1115.

88. Coulter Electronics Inc. (1983) EASY I User's Manual

89. Cass, C.E., Dahlig, E., Lau, E.Y., Lynch, T.P., & Paterson, A.R.P. (1979) Fluctuations in Nucleoside Uptake and Binding of the Inhibitor of Nucleoside Transport, Nitrobenzylthioinosine, during the Replication Cycle of HeLa Cells. *Cancer Res.* 39, 1245-1252.

## Appendix: Cell Cycle Analysis.

Cell cycle analysis consists primarily of the division of the DNA histogram generated using the flow cytometer, into several compartments followed by the calculation of the percentage of total cells in each compartment [88]; these compartments are the $G_1$, S and $G_2M$ phases of the cell cycle; the S compartment may be further subdivided into four sub-compartments *i.e.*, early S, mid $S_1$, mid $S_2$ and late S. Cells in $G_2$ phase and in M phase were analysed together because the DNA content of cells in these two phases is the same. In the computer analysis of the cell cycle, several assumptions concerning the distribution of cells in the cycle are made: i) cells in the $G_1$ phase form a gaussian distribution at some mean DNA content, ii) the $G_2M$ cells also form a gaussian distribution but at twice the DNA content of the cells in $G_1$ phase, and iii) cells in the S phase form a continuous distribution between $G_1$ and $G_2M$ peaks.

The Coulter Electronics EASY I software for cell cycle analysis consists of three separate programs. In the first program, Para 1, the peak positions of three peaks, usually $G_1$, $G_2M$ and a marker, *i.e.*, chick red blood cells, are located and calculations performed to find the mean position and standard deviation of each peak. Based on the assumptions stated above, the percentage of cells in each of the three primary phases, *i.e.*, $G_1$, S and $G_2M$, are also calculated.

The second and third programs, Modset and Para 2, carry out a two part analysis. All the information needed for the mathematical evaluations are set in Modset, *e.g.* the basic assumptions as stated at the begining of the section and in addition to that, positions of the four sections of S phase relative to $G_1$ and $G_2M$; the parameters for calculations; and the equations.

The main purpose of Modset is to create a 'model' which the computer can use in Para 2 analysis. A 'model' is essentially the raw DNA histogram (generated on the cell sorter) with its compartment positions defined, *i.e.*, the histogram has been subdivided into $G_1$, early S, mid $S_1$, mid $S_2$, late S and $G_2M$ sections. To do this, the computer uses the value for $G_1$ peak

position (which was assigned to it based on results from Para 1 analysis) to decide where the $G_2M$ should be (approximately double the DNA content away), followed by division of S into four sections.

If the population of cells is highly perturbed and if, for example, the $G_1$ peak has almost disappeared, then the position of this peak can be estimated using the value for the position of the marker peak in the perturbed sample (from Para 1 analysis) and multiplying this by the $G_1$ peak: marker peak ratio of the control populations (from Para 1 analysis). The peak position of the marker in relation to $G_1$ peak should remain unchanged in all samples.

It is in this program in which the computer is given either the option to find the optimal compartment position values for the model using the assigned values as initial estimates, or to use the assigned values for its final calculations of the distribution of cells in the cell cycle. Usually the former option is used in analysis of control samples and the latter in drug-treated samples.

The actual analysis is performed by Para 2. Here, the optimum peak height for the specified position of the $G_1$ peak of the model and for the remaining compartments are calculated. Next, the chi square test is performed on the calculated data, to determine the quality of fit of the calculated data to the model. If given the first option mentioned above, then these calculations are repeated with altered $G_1$ peak position (by the computer). When certain pre-set limits are reached, this search for best-fit data was terminated. The final calculated values for the model are then used in calculating the percentage of the total cells in each phase of the cell cycle.

**Accuracy of analysis.**

A population of perturbed cells might not obey the assumptions made and hence the final calculated optimal heights of each compartment may be inaccurate. The quality of fit of the calculated data to the raw data can be graphically visualised; three examples are shown in Fig. A.1. A brief explanation of the graphs in Fig. A.1: in the top graph, the shaded histogram

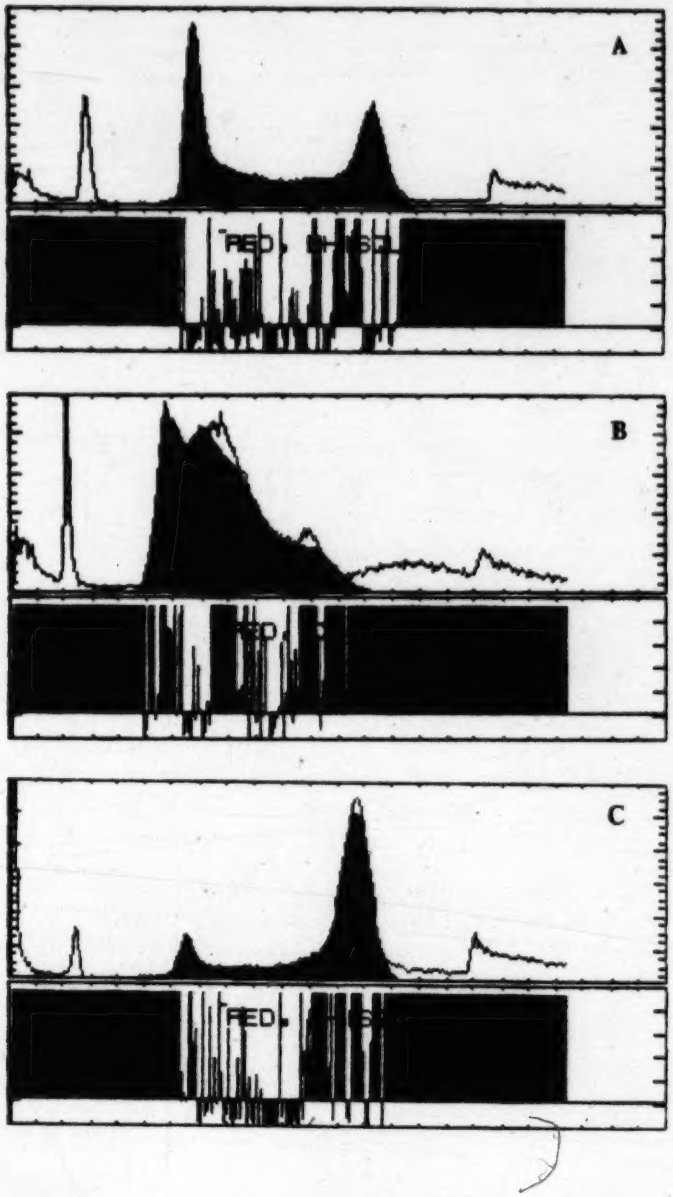



**Fig. A.1 Cell cycle histograms and chi-square plots of control and drug-treated cell populations** (A, control for 5HP at 16h; B, 2.0 mM IMPY at 16h; C, 200 $\mu$M INOX at 16h).

represents the calculated data and the unshaded represent the raw data; in the second plot of reduced chi square values, shading above the solid line indicates poor fitting of the data (underfitting) and shading below the line indicates that calculated data were too flexible (overfitting), *i.e.*, they can fit statistical noise as well as true biological signals.

In the analysis of control samples, in which the population of cells is asynchronous, the assumptions made were usually true and hence the calculated values were close to the actual values. Fig. A.1A shows the chi square plot for a control sample; note the small amount of shading above the solid line. In synchronised cells where the number of cells in $G_2M$ was low, the model tends to underfit the actual data and therefore inaccurately calculated percentages result. Fig. A.1B shows the high reduced chi square values around the $G_2M$ region. This figure also demonstrates that the assumption of continuous distribution of cells in S phase may not hold in some cases of perturbed population of cells. However, cells with a low $G_1$ populations have a different problem, in that the model fits the $G_1$ peak well but underfits the $G_2M$ peak (Fig. A.1C). This is because in Para 2 analysis, the position of the $G_2$ peak is fixed in relation to $G_1$ and therefore, this presumed position may not be the highest point of the $G_2M$ peak.

One must consider the limitations when analysing the results generated by these programs. Despite its limitations, however, the program has its advantages: it can evaluate mildly perturbed and control cells accurately, or even highly perturbed cells if their distribution obeys the assumptions, and it can perform the very complicated, tedious mathematical evaluations at a much faster rate than any individual can.